Note to copy editor: Standard Methods Chapter format

Robust induction of DARPP32 expressing GABAergic striatal neurons from human pluripotent stem cells

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Running Head: Derivation of MSNs from hPSCs

Abstract

Efficient generation of disease relevant neuronal subtypes from human pluripotent stem cells (PSCs) is fundamental for realising their promise in disease modelling, pharmaceutical drug screening and cell therapy. Here we describe a step-by-step protocol for directing the differentiation of human embryonic and induced PSCs (hESCs and hiPSCs, respectively) towards medium spiny neurons, the type of cells that are preferentially lost in Huntington's disease patients. This method is based on a novel concept of Activin A-dependent induction of the lateral ganglionic/striatal fate using a simple monolayer culture paradigm under chemically defined conditions. Transplantable medium spiny neural progenitors amenable for cryopreservation are produced in less than 20 days, which differentiate and mature into a high yield of dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa (DARPP32) expressing gamma-aminobutyric acid (GABA)-ergic neurons *in vitro* and in the adult rat brain after transplantation. This method has been validated in multiple hESC and hiPSC lines, and is independent of the regime for PSC maintenance.

Key words: Activin, DARPP32, lateral ganglionic eminence, medium spiny neuron, pluripotent stem cell, neural differentiation, Huntington's disease, striatum, transplantation.

1 Introduction

Preferential loss of the γ-amino butyric acid (GABA)-ergic medium-sized spiny neurons (MSNs) in the striatum is the primary pathology of Huntington's disease (HD) ***(1)***. Whilst it is known that MSN degeneration is caused by an extended CAG-repeat mutation in the Huntingtin (*HTT*) gene, the cellular and molecular mechanism by which mutant HTT protein triggers striatal cell death remains largely unknown. Consequently, there is currently no cure for HD. Human MSNs generated *in vitro* from healthy pluripotent stem cells (PSCs) offer great promise for developing cell-based therapy for treating HD, while those derived from patient cells or engineered to carry the mutant *HTT* gene would serve as a useful platform for understanding the aetiology of HD and drug discovery therein.

1.1 Generation of striatal neurons from pluripotent stem cells

We have recently reported a highly reliable generation of MSNs from human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) ***(2)*****.** The method is a simple monolayer differentiation procedure using a single chemically defined basal medium formula throughout. Unique to this protocol is the application of Transforming Growth Factor-β (TGF-β) family protein Activin A (referred to hereafter as activin) during a defined window of neural conversion. Previous studies showed the presence of activin subunits, receptors and their transcriptional effectors, the Smad proteins, in the developing subpallium ***(3,4)***, suggesting potential regulation of striatal development by TGF-β family signalling. In line with these findings, we subjected hPSC-derived forebrain progenitors to activin treatment to induce an MSN fate specification ***(2)***. This resulted in a rapid upregulation of CTIP2 (also known as BCL11B), a transcription factor required for MSN development *in vivo* and the only transcription factor reported capable of directly programming skin

fibroblasts into functional MSNs ***(5,6)***. We also demonstrated that neural progenitors obtained using this protocol are committed to a striatal fate that exhibit characteristics of the lateral ganglionic eminence (LGE) such as expression of specific markers including *NOLZ1* and *FOXP2*. Furthermore, induction of these striatal transcripts by activin can be completely blocked by a specific activin/SMAD inhibitor, SB431542, suggesting that the specification of an LGE-derived MSN fate in this system requires the TGF-β family signalling via activation of the SMAD2/3 pathway.

Sonic hedgehog (SHH)-based morphogen signalling has been exploited previously for generating dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa (DARPP32) expressing MSNs from hESCs ***(7-9)***. Resulting MSN progenitors expressed pan-GE markers *FOXG1*, *OTX2* and *GSX2*. However, LGE-specific transcription factor *CTIP2* was unaffected and other LGE markers were not analysed at progenitor stage. In contrast, our studies on multiple hPSC lines support the notion that activin preferentially induces LGE/MSN characteristics whilst SHH elicits a generic ventralising effect with no apparent activity on LGE-specific gene expression. Technically, the current method implements a simple monolayer differentiation paradigm without the need for embryoid body or aggregate formation used in earlier studies, which in our hands tend to generate more heterogeneity and is less reproducible. Moreover, compared to the others, our method takes a shorter time to produce LGE progenitors, the likely target for a cell therapy product, and postmitotic DARPP32^{+} MSNs.

For most of the hPSC lines tested, our method requires no passaging for obtaining neural progenitors committed to a striatal fate that exhibit characteristics of the LGE; and hence just one passaging step is necessary to facilitate terminal differentiation of the neural progenitors towards MSNs and their maturation and survival. So far, the

protocol has been shown to be effective in five hESC lines and two hiPSC lines, including the research stock of good manufacturing practice (GMP) grade RC9 and RC17 hESCs (http://www.nibsc.org). Moreover, the protocol performs well independently of the culture conditions of hPSC lines, i.e. whether the cells were routinely maintained on feeders or in feeder-free conditions, the supporting matrix the cells were grown on, the types of culture media used for maintaining self-renewal or dissociation methods implemented to passage hPSCs. Additionally, LGE progenitors generated are sufficiently robust to survive cryopreservation for future experiments. Once thawed, they readily undergo terminal differentiation into MSNs *in vitro* or can be transplanted into the lesioned animal striatum.

1.2 Experimental Design

Maintenance of hPSC cultures in feeder-free conditions. This protocol is written with feeder-free hPSC (ESC or iPSC) cultures as the starting material, although it also applies to hPSCs maintained on feeders. For the latter, an extra step of plating before seeding for differentiation is required to remove the feeder cells (*see* **Note 1**). However, for a better and reliable yield of MSNs, it is advisable to wean the hPSC lines off feeders.

Monolayer differentiation of hPSC cultures towards LGE progenitors. An overview of the experimental procedure is given in **Figure 1a**. Derivation of LGE progenitors from hPSC cultures is a technically simple procedure that consists of two main stages. Firstly, pluripotent cells are converted into forebrain progenitors with a cocktail of SMAD inhibitors and then patterned towards an LGE fate with activin. Refer to **Figure 1b** for characteristic morphology of intermediate progenitors at each stage of the protocol.

Insert Figure 1 about here

The first stage of differentiation entails efficient neural conversion of hPSCs within nine days under adherent culture conditions using a modified dual-SMAD inhibition protocol ***(10,11)***. In contrast to Chambers et al. who gradually replace media supplemented with knockout serum replacement with that of N2 during the early phase of neural fate conversion, we begin the neural induction with N2B27 medium and use this single medium formula throughout the protocol (*see* **Cell Culture Reagents and Setup** for a detailed medium composition). A relatively high cell density at the onset of differentiation is optimal for efficient induction of forebrain progenitor fate. Crucially, we use retinol-free B27 for the first 20 days to reduce caudalisation and maximise subsequent LGE patterning.

Induction of the LGE fate in FOXG1 expressing forebrain progenitors is achieved by using activin as the exogenous patterning factor. This step generally starts from day 9, or day 10 for cultures that need a passage step at day 9, by replacing the SMAD inhibitors with activin. Refer to **Figure 1b** for characteristic morphology of differentiating LGE progenitors. Rosettes can be visible in cultures re-plated at day 9 but these structures may not be apparent in cultures without this extra passage. It is advisable to include a no activin control in the experimental design. These neural progenitors will adopt a dorsal telencephalic fate, demonstrated by their expression of PAX6 and DMRT5, and later give rise to cortical pyramidal neurons.

LGE progenitors derived from this stage of the protocol may be analysed by immunofluorescence (IF) staining and qRT-PCR, re-plated and terminally differentiated into MSNs (**Figure 2**), or transplanted into the lesioned animal striatum for further analyses. They also have the advantage over earlier progenitors of being robust enough to be cryopreserved for future experiments. Frozen stocks of LGE

neural progenitors remain viable long-term when stored in liquid nitrogen. Upon thawing, LGE progenitors can be terminally differentiated into MSNs *in vitro* or transplanted into an animal model of HD (**Figure 3**).

Insert Figures 2 and 3 about here

2 Materials

2.1 Cell Culture Reagents and Setup

1. hPSC culture medium: mTeSR™1, TeSR™-E8™ Kit for hESC/hiPSC Maintenance (E8 medium) or StemPro® hESC SFM Kit.
2. Cell culture substrates: Matrigel® Matrix hESC-qualified, CELLstart™ CTS™ Substrate (see **Note 2**), Matrigel® Growth Factor Reduced Basement Membrane Matrix (GFR Matrigel), Human Plasma Fibronectin Purified Protein (Fibronectin), Laminin, and Poly-D-lysine hydrobromide (PDL).
3. Cell dissociation reagents: EDTA 0.02% solution for cell culture applications and Accutase.
4. Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium (DPBS -Ca-Mg) for cell culture applications
5. N2B27 complete differentiation medium (150 mL): 100 mL of DMEM/F-12 medium, 50 mL of Neurobasal medium, 1.5 mL of 200 mM L-Glutamine, 1.5 mL of 10,000 U/mL Penicillin/Streptomycin, 1 mL of 100X N-2 supplement (supplemented 1:10 with 7.5% Bovine Serum Albumin), 1 mL of 50X B-27 supplement (with/without vitamin A), and 150 µL of 50 mM 2-Mercaptoethanol solution [CAUTION: 2-Mercaptoethanol is toxic if inhaled, ingested or spilled on skin. Wear gloves and lab coat and handle it with care under a chemical

cabinet.]. Mix N2B27 medium throughout and store at 4 °C. For best effect, it is advisable to use the complete N2B27 medium within a month.

6. Neural induction medium A: complete N2B27 medium without vitamin A, 100nM LDN-193189, 200 nM Dorsomorphin dihydrochloride, 10 µM SB 431542. Complete medium with factors can be kept at 4 °C for several days but use fresh medium if there is any noticeable decrease in cell growth rate.
7. Neural induction medium B: complete N2B27 medium without vitamin A, 100nM LDN-193189, 200 nM Dorsomorphin dihydrochloride.
8. LGE patterning medium: complete N2B27 medium minus vitamin A, 25 ng/mL recombinant human Activin A.
9. Terminal differentiation medium: complete N2B27 medium with vitamin A, 10 ng/mL recombinant human BDNF, 10 ng/mL recombinant human GDNF.
10. Y-27632 RHO/ROCK pathway inhibitor (ROCK inhibitor)
11. Cell freezing medium: Cell culture medium supplemented with 10% (vol/vol) dimethyl sulfoxide (DMSO) or CryoStor® CS10 Cryopreservation Medium (STEMCELL Technologies).

2.2 IF Staining

1. PBS buffer: deionized water, 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4 at 25 °C.
2. Cell fixation solution: 3.7% (wt/vol) paraformaldehyde solution in PBS [CAUTION: Paraformaldehyde is extremely toxic and must be handled under a chemical cabinet. Gloves and lab coat should be worn to avoid contact with skin.]
3. 0.3% PBST buffer for immunofluorescence staining: 1L PBS, 3 mL Triton X-100.

4. Blocking buffer for IF staining: 1% (wt/vol) BSA and 3% (vol/vol) donkey serum solution in PBST.
5. Hoechst 33258, 10 mg/mL solution in water
6. Dako Fluorescent Mounting Medium
7. A summary of antibodies we routinely use is presented in **Table 1**

Insert Table 1 about here

2.3 RNA extraction, RT-PCR and qRT-PCR

1. TRI Reagent [CAUTION: TRI Reagent is extremely toxic and must be handled under a chemical cabinet. Gloves and lab coat should be worn to avoid contact with skin.]
2. Chloroform [CAUTION: Chloroform is toxic if inhaled, ingested or spilled on skin. Wear gloves and lab coat and handle it with care under a chemical cabinet.]
3. Ethanol [CAUTION: Ethanol is toxic if inhaled, ingested or spilled on skin. Wear gloves and lab coat and handle it with care under a chemical cabinet.]
4. DEPC-treated water
5. SuperScript III First-Strand Synthesis System (Life Technologies)
6. iQ™ SYBR® Green Supermix (Bio-Rad)
7. The sequence information for validated PCR primers is summarised in **Table 2**

Insert Table 2 about here

2.4 Equipment

1. 15, 30 & 50 mL conical centrifuge tubes
2. 10, 20, 200 & 1000 µl sterile pipette tips

3. 2, 5, 10, 25 & 50 mL sterile serological pipettes
4. Electronic pipettor
5. 6-, 12-, 24-well Nunc™ Cell-Culture Treated Multidishes
6. 1.8 mL Sterile Cryogenic Vials
7. 250 mL Solution Bottle
8. ⌀ 13 mm Glass coverslips
9. Glass Microscope Slides
10. 1.5 mL RNase-free Screw-Cap Microcentrifuge Tubes
11. 96-well PCR plate
12. Mr. Frosty™ Freezing Container
13. Laminar Flow Biosafety Cabinet
14. Humidified 5% CO_2 37°C Incubator
15. Waterbath
16. Centrifuge 5810 R
17. Phase-contrast Inverted Microscope
18. Plate vortexer
19. Rocker
20. Fluorescence Microscope
21. Centrifuge 5424 R
22. BioSpectrometer basic
23. Thermocycler
24. Chromo4 System (Bio-Rad)

3 Methods

3.1 Preparation of hPSC Culture for Neuronal Differentiation in Feeder-free Conditions

1. Every lot of Matrigel® Matrix hESC-qualified matrix (Matrigel) can have a slightly different concentration of proteins, thus the suggested dilution ratio will vary from batch to batch and will be indicated on the datasheet. Dilute Matrigel in DMEM/F-12 as suggested and coat 6-well culture plates at the volume of 1 mL/well. Gently shake the plate to cover all the surface area with the coating solution and transfer to a humidified 5% CO_2 37 °C incubator for at least 1 hour. When this is ready, transfer Matrigel to new wells (can be re-used once) and wash the coated wells once with DPBS -Ca-Mg before plating cells.
2. Thaw hPSCs and plate in E8 medium (*see* **Note 3**) at a density of 0.75 - 1 x 10^6 cells per well of a 6-well plate for a successful outcome.
3. Expand if necessary and grow hPSC cultures to 70 – 80% confluence prior to seeding cells for a differentiation. Typically, two wells of a 6-well plate of hPSCs are required for seeding one full 6-well or 12-well plate for differentiation at a density of 70,000 – 100,000 cells/cm^2 (*see* **Note 4**).
4. On the day of seeding cells for a differentiation, pre-coat culture containers with GFR Matrigel (*see* **Note 5**). For a thin coating method dilute 1:15 GFR Matrigel in ice cold DMEM/F12, mix throughout and add 0.5 mL/well into each well of a 12-well plate or 1 mL/well into each well of a 6-well plate. Coat culture vessels on the day of use in a humidified 5% CO_2 37 °C incubator for 1 – 1.5 hours. Aspirate unbound material from coated wells and rinse gently once using DPBS -Ca-Mg. Plates are now ready to use. Use 6-well plates for large-scale experiments (e.g. transplantation or banking of LGE progenitors) and 12-well plates for small-scale experiments and qRT-PCR and IF staining analyses.

5. Aspirate old culture medium from the cells to be used and then gently wash wells with 2 mL of DPBS -Ca-Mg.
6. Remove DPBS -Ca-Mg from culture vessels and add 1 mL of Gentle Cell Dissociation Reagent or EDTA 0.02% solution to each well and incubate at 37 °C 5% CO_2 for 2 – 5 minutes. If using EDTA, treat cells for 2 minutes at 37°C 5% CO_2. Incubation time may vary among different cell lines and depending on the coating matrix in use. Therefore, inspect the plates under a microscope and stop the reaction when edges of the colonies start to pull away from the plate and white gaps appear between cells.
7. Aspirate the dissociation medium leaving the cells attached to the bottom of the well and immediately add 3 mL of culture medium to the cells. Some detachment of cells upon addition of culture medium is normal at this stage.
8. Using a 10 mL serological pipette, very gently scratch the bottom of the well to dislodge cells in small clumps (50 – 100 μm). Not all cells will detach, this is normal.
9. Collect most of the cells from the well and transfer them to a 30 mL universal tube. Dilute collected cell suspension with hPSC culture medium to achieve a seeding density of 70,000 – 100,000 cells/cm^2. Gently dissociate cells into small clusters by carefully pipetting 2 – 3 times with a serological pipette (*see* **Note 6**).
10. Seed cells for a differentiation into pre-coated culture vessels (*see* **Note 1**). The final volume of medium should be 1 mL/well for 12-well plate or 3 mL/well for 6-well plate. Transfer culture containers to the 37 °C 5% CO_2 incubator and be sure to gently move the plates in several quick, short, side-to-side and back-and-forth motions to obtain even distribution of cells.
11. Inspect the hPSC cultures the next morning under a microscope. Cells should have attached as small undifferentiated colonies in a monolayer evenly

distributed across the wells (*see* **Note 7**). Slightly swirl the plate immediately before feeding hPSCs to collect dead cells and debris in the middle of culture vessels. Aspirate old medium with dead cells and replace it with 1.5 mL/well for 12-well plates or 3 mL/well for 6-well plates of pre-warmed fresh hPSC culture medium.

12. Incubate the plates at 37 °C with 5% CO_2. Feed cells daily until cultures reach 80 – 90% confluence, at which point move to the next stage. Aim to start differentiation 2 – 3 days after seeding cells. If it takes cultures longer to grow 80 – 90% confluent, seed higher cell concentrations.

3.2 Monolayer Differentiation of hPSCs to Forebrain Progenitors

1. Once hPSC cultures grow to 80 – 90% confluent, aspirate all hPSC culture medium and gently wash cells once with DPBS -Ca-Mg. Do not wait until hPSC cultures grow 100% confluent before starting a differentiation as this increases cell death and leads to poor neural induction.
2. Remove DPBS -Ca-Mg from culture vessels and add pre-warmed neural induction medium A. The final volume should be 3 mL/well for a 6-well plate or 1.5 mL/well for a 12-well plate. This day is designated as Day 0 (D0) of differentiation. Incubate the plates in the humidified 37 °C 5% CO_2 incubator. Depending on a cell proliferation rate, either daily or every other day swirl the plate gently immediately before feeding to collect dead cells and debris in the middle of the well. Replace ⅔ of old medium with fresh pre-warmed medium. Inspect differentiating cultures under a microscope every day and observe changes in cell morphology towards a round shape, reduction in size and nucleus-to-cytoplasm ratio, all of which are characteristic features of cells exiting

a pluripotent stage and committing to a telencephalic fate as shown in **Figure 1b**. Keep differentiating cells in neural induction medium A until D5.

3. On D5 of differentiation, feed cultures with neural induction medium B. Keep cells in neural induction medium B until D9. Feed the cultures either daily or every other day depending on the density and growth rate of differentiating cells.
4. D9 of differentiation signifies the end of neural induction phase. Refer to **Figure 1b** for representative cellular morphology of cultures committed to the neuroectoderm. To characterise forebrain progenitors at this stage, cells can be fixed for IF staining and lysed to extract RNA for qRT-PCR analysis. If differentiating cells require passaging onto Fibronectin-coated plates at this stage follow steps in ***3.3***, alternatively proceed to ***3.4***.

3.3 Passage of Forebrain Progenitors onto Fibronectin-coated Culture Vessels

1. For a successful outcome, some cell lines (e.g. H7, H9) differentiating on GFR Matrigel require passaging once they start forming multi-layered cultures during D8-12 of differentiation (*see* **Note 8**). Otherwise, forebrain precursors become stressed, start to detach from the matrix and fail to differentiate into LGE progenitors. Also, with some batches of CELLstart, neuroectodermal-like cells should be passaged at this stage or they will detach from the matrix and die.
2. On the day of passage, coat culture vessels with Fibronectin solution (15 µg/mL in DPBS -Ca-Mg) for at least 1 hour in a humidified 5% CO_2 37 °C incubator. Use 6-well plates for large-scale experiments (e.g. transplantation or banking of LGE progenitors) and 12-well plates for small-scale experiments and qRT-PCR and IF staining analyses.
3. Depending on what day of differentiation forebrain progenitors are being passaged on, prepare the volume of neural induction medium B or LGE

patterning medium supplemented with 10 µM ROCK inhibitor enough to passage cells at a 2:3 ratio based on the area of culture containers plus extra 2 – 3 mL and pre-warm to room temperature. The final volume for seeding forebrain progenitors should be 2 mL/well for 6-well plates or 1 mL/well for 12-well plates.

4. Discard ⅔ of old medium from each well and replace it with fresh medium supplemented with ROCK inhibitor from the previous step. Incubate cells for at least 1 hour at 37 °C 5% CO_2.
5. Following ROCK inhibitor treatment, collect ⅔ of medium from each well into a 50 mL centrifuge tube. Aspirate the remaining medium and gently wash cultures once with DPBS -Ca-Mg.
6. Discard DPBS -Ca-Mg and add 1 mL/well for 6-well plates or 0.5 mL/well for 12-well plates of EDTA 0.02% solution. Incubate cells in EDTA for 1 – 2 minutes at 37 °C 5% CO_2 until small gaps between cells become visible under a light microscope (*see* **Note 9**). Aspirate EDTA and, without washing cells, add previously collected medium back into each well. Using a 10 mL serological pipette, very gently scratch the bottom of the well to dislodge cells in large clumps (300 – 600 µm). Not all cells will detach, this is normal.
7. Collect most of the cells from all wells within the same experimental group and transfer them to a 50 mL centrifuge tube. Dilute the cell suspension with fresh medium to the required volume as determined in Step 3. Aspirate Fibronectin solution from coated culture vessels and discard. It is not necessary to rinse coated wells before seeding cells. Using a serological pipette, very gently mix the cell suspension by carefully pipetting 1 – 2 times and seed cells into pre-coated wells in 2 mL/well for 6-well plates or 1 mL/well for 12-well plates.
8. Transfer the plates to a humidified 37 °C incubator with 5% CO_2 and ensure cells are distributed evenly across wells by gently moving the plates in several quick,

short, side-to-side and back-and-forth motions. On the next day, proceed to the next stage.

3.4 Patterning of Forebrain Progenitors towards the LGE Fate

1. On D9 of differentiation, prepare fresh LGE patterning medium to feed cells with 1.5 mL/well for 12-well plates or 3 mL/well for 6-well plates and pre-warm to room temperature. It is important to have culture vessels with forebrain progenitors that are not being treated with activin to use as a negative control in future analyses. Keep control cultures in N2B27 medium minus vitamin A.
2. Remove most of old medium from each well leaving just a thin film of liquid to cover cells and add pre-warmed LGE patterning medium. Incubate plates at 37 °C 5% CO_2.
3. Inspect the cells daily and replace ⅔ of the LGE patterning medium every other day with fresh pre-warmed medium until D20. Refer to **Figure 1b** for representative cellular morphology of neuronal progenitors at this stage.
4. D20-22 of differentiation signify the end of the LGE patterning phase. LGE progenitors, the end product of this stage of the protocol, can be characterised by IF staining and qRT-PCR, re-plated onto PDL/Laminin and terminally differentiated into MSNs *in vitro*, transplanted into an animal model of HD for further analysis or cryopreserved for future experiments.

3.5 Terminal Differentiation of LGE Progenitors into MSNs

1. For the terminal differentiation into MSNs *in vitro*, passage LGE progenitors on D20-22 of differentiation at a split ratio of 1:1 – 1:5 based on the area of culture vessels. One day before the passage, start preparing the required number of PDL/Laminin-coated culture vessels. Use 12-well plates for qRT-PCR analysis

and glass coverslips in 24-well plates for IF staining. Dilute PDL in DPBS -Ca-Mg to 10 µg/mL concentration and apply coating solution at a final volume of 0.25 mL for 24-well plates or 0.5 mL for 12-well plates. Coat tissue culture vessels with PDL in a humidified 5% CO_2 37 °C incubator for at least 2 hours. After this, discard PDL coating solution and wash coated culture containers with DPBS -Ca-Mg. Dilute Laminin in DPBS -Ca-Mg to 10 µg/mL concentration and apply coating solution overnight in a humidified 5% CO_2 37 °C incubator. When this is done aspirate Laminin solution from culture vessels and discard. It is not necessary to rinse coated wells before seeding cells.

2. Remove the medium from cultures and gently wash cells once with DPBS -Ca-Mg. Discard DPBS -Ca-Mg and incubate cells in EDTA 0.02% solution for 2–3 minutes at 37 °C 5% CO_2.
3. Aspirate EDTA and, without washing cells, add 2 mL/well for 6-well plates or 1 mL/well for 12-well plates of pre-warmed LGE patterning medium. Using a 1000 µL pipette tip, very gently scratch the bottom of the well to dislodge cells. LGE progenitors are quite robust and can be collected in very small clumps (up to quasi-single cell suspension) at this stage.
4. Collect most of the cells from all wells within the same experimental group and transfer them to a 50 mL centrifuge tube. Dilute the cell suspension in the required volume of pre-warmed LGE patterning medium to seed cells in 1 mL/well for 12- and 24-well plates. Seed LGE progenitors into the culture container and transfer the plate to a humidified 5% CO2 37 °C incubator and ensure cells are distributed evenly across wells by gently moving the plate in several quick, short, side-to-side and back-and-forth motions.
5. On the following day, pre-warm enough terminal differentiation medium to feed cells with 1.5 mL/well for 12-well plates or 1 mL/well for 24-well plates. Gently

swirl the plate immediately before feeding to collect dead cells and debris in the middle of the well. Aspirate most of the old medium from each well leaving just a thin film of liquid to cover cells, add fresh pre-warmed medium and return the plates to the humidified 5% CO_2 37 °C incubator. Every other day or as needed, feed cells with terminal differentiation medium until the end of the experiment by replacing ⅔ of old culture medium per well with pre-warmed fresh medium. Neurons can be maintained in culture for over 3 months, although we tend to do most of our initial IF staining (**Figures 2 and 3**) and qRT-PCR analyses on D35-40 of differentiation.

3.6 Characterisation of cells in vitro

The following options describe processing of cell cultures to produce fixed cells or RNA, as required for further analysis. To fix cells and perform IF staining, follow option A. To extract RNA from cells and perform qRT-PCR, follow option B.

3.6.1 Fixation of cultures and IF staining

1. Wash cells gently once with DPBS -Ca-Mg and discard the liquid.
2. Add 250 µL/cm^2 of cold 3.7% (wt/vol) PFA solution per well and incubate cells at 4 °C for 15 minutes.
3. Remove PFA solution and place it in a hazardous waste container [CAUTION: Paraformaldehyde is extremely toxic and must be handled under a chemical cabinet. Gloves and lab coat should be worn to avoid contact with skin.].
4. Wash cells gently three times with DPBS -Ca-Mg. Avoid drying out cells between washes. Fixed cultures can be stored at 4 °C for at least 2 weeks

before proceeding with the staining. Tightly wrap the plates to avoid evaporation of DPBS.

5. Wash cells gently three times with PBST for 10 min per washing.
6. Add 250 µL/cm^2 of blocking buffer and incubate at room temperature for 20 min – 1 h.
7. Without washing, transfer cells to 125 µL/cm^2 of primary antibody solution (**Table 1**) in blocking buffer and incubate at room temperature for 2 h with gentle rocking. This step can be extended to overnight at 4 °C with gentle rocking.
8. Wash cells gently three times with PBST for 10 min per washing.
9. Add 125 µL/cm^2 of Alexa-Fluor secondary antibody solution (1:200) in blocking buffer and incubate at room temperature in the dark for 1 h with gentle rocking.
10. Wash cells gently three times with PBST for 10 min per washing.
11. Incubate cells in Hoechst solution (1:10,000) in PBST for 5 minutes with gentle rocking.
12. Wash cells gently three times with PBS for 10 min per washing.
13. Add a drop of Fluorescent mounting medium to a glass microscope slide. To avoid air bubbles, touch a coverslip with cells to the mounting medium at an angle and slowly lower the coverslip until fully on slide. Mount only a few coverslips at a time as the Fluorescent mounting medium dries out fast.
14. Capture the fluorescence images with a confocal microscope and quantify markers by manually counting cells from randomly selected fields.

3.6.2 RNA extraction and qRT-PCR

Extraction from 1 well of a 12-well plate yields enough RNA for qRT-PCR analyses.

1. Wash cells gently once with DPBS -Ca-Mg and discard the liquid.
2. Add 0.5 mL/well of TRI Reagent® and resuspend the culture by repeatedly pipetting up and down [CAUTION: TRI Reagent® is extremely toxic and must be handled under a chemical cabinet. Gloves and lab coat should be worn to avoid contact with skin.].
3. Transfer the resulting lysate into an RNase-free microcentrifuge tube and place it on ice. The resulting lysate can be directly processed or stored at -80 °C for several months.
4. Perform RNA extraction using TRI Reagent® protocol according to the manufacturer's recommendations. Measure the RNA concentration using the Eppendorf BioSpectrometer® basic. The resulting RNA can be directly processed or stored at -80 °C for several months.
5. Synthesise cDNA according to the manufacturer's recommendations using SuperScript® III first-strand synthesis system and random primers. Use the same total amount of RNA for each sample. Synthesised cDNA can be directly processed or stored at -20 °C until use.
6. Carry out qRT-PCR according to the manufacturer's instructions using iQ™ SYBR® Green Supermix and a Chromo4 System. Use genes encoding β-Actin and GAPDH as reference genes and normalise data to the basal conditions. The sequence information for all PCR primers used in our initial publication is summarised in **Table 2**.

3.7 Preparation of the Cell Suspension for Transplantation of LGE Progenitors into an Animal Model of HD

1. On D20-22 of differentiation, remove the medium from cultures and gently wash cells once with DPBS -Ca-Mg. Aspirate DPBS -Ca-Mg from cells and add 1

mL/well for 6-well plates or 0.5 mL/well for 12-well plates of Accutase Cell Dissociation Agent. Incubate cultures in dissociation agent for 10 minutes at 37 °C 5% CO_2. Inspect the plates after first 5 minutes and look for floating cells. Gently pipette the dissociation agent over the surface of the culture 3 times and reject 3 times. Return the plates to 37 °C 5% CO_2 for 5 more minutes.

2. Gently pipette the dissociation agent over the surface of the culture to detach and dissociate all cells. Check under a light microscope to confirm that cultures are in a quasi-single cell suspension. If not, return the plates to 37 °C 5% CO_2 for 5 more minutes. Do not keep cells in the dissociation reagent for more than 30 minutes in total, as this decreases cell survival and compromises a successful outcome of the experiment.
3. Collect the cells in the dissociation agent from all wells within the same experimental group into a 50 mL centrifuge tube. Set aside a small aliquot of the cell suspension to count cells using trypan blue. Add LGE patterning medium to the 50 mL tube with the cell suspension at a ratio 4:1 to dilute out the enzymes and stop dissociation. Centrifuge at 250 g for 3 minutes. In the meantime, count cells in the cell suspension that was set aside.
4. After centrifugation is completed, aspirate the supernatant from the centrifuge tube with a vacuum pump without disturbing the cell pellet. Disrupt the cell pellet by gently flicking the tube. Resuspend LGE progenitors in DMEM/F-12 at the required concentration (e.g. 1–2.5 x 10^5 cells/µl) and proceed with the transplantation into an animal model of HD.

3.8 Cryopreservation of LGE Progenitors

1. Prior to freezing LGE progenitors, fill the lower chamber of a Mr. Frosty™ Freezing Container with isopropanol and pre-chill the container at 4 °C. This system is designed to achieve the optimal rate of cooling for cell preservation, approximately -1 °C/minute.
2. On D20-22 of differentiation, LGE progenitors can be frozen either as a single cell suspension or as small clusters of cells. To obtain a single cell suspension and count cells follow steps in ***3.7*** and proceed to the next step. To collect LGE progenitors in small clusters of cells follow steps in ***3.5*** and proceed to the next step.
3. Centrifuge at 250 g for 3 minutes. After centrifugation is completed, aspirate the supernatant from the centrifuge tube with a vacuum pump without disturbing the cell pellet. Disrupt the cell pellet by gently flicking the tube.
4. Cells can be frozen either in CryoStor® CS10 or in 10% (vol/vol) DMSO solution in the LGE patterning medium. If freezing LGE progenitors as a single cell suspension, resuspend collected cells in cold cryopreservation medium at the concentration of 1–10 x 10^6 cells/mL. If freezing cultures as small clusters of cells, resuspend collected LGE progenitors in cold cryopreservation medium to a final volume of 2 mL per well of 6-well plate or 1 mL per well of 12-well plate to be frozen.
5. Add 1 mL of cell suspension to each cryovial and immediately put the vial in the freezing container. Transfer the Mr. Frosty™ Freezing Container with cells to a -80 °C freezer.
6. On the next day, put cryovials in the short-term storage at -80 °C or long-term storage in a liquid nitrogen tank. LGE progenitors can be stored for up to 6 months at -80 °C and for years in the liquid nitrogen storage tank.

3.9 Thawing LGE Progenitors for Downstream Applications

1. If thawing LGE progenitors for the terminal differentiation into MSNs *in vitro*, prepare the required volume of LGE patterning medium and pre-warm to room temperature. The day before thawing cells, start preparing culture vessels or glass coverslips coated with PDL/Laminin as described in ***3.5***. The typical density at which LGE progenitors should be seeded is 0.5–1 x 10^6 cells/cm^2 in 1 mL/well for 12- and 24-well plates.
2. Pre-warm a water bath to 37 °C. Aliquot 10 mL of LGE patterning medium per vial of cells to be thawed into a 15 mL centrifuge tube and warm to 37 °C in a water bath. This is the **wash** media.
3. Remove cryovials of LGE progenitors from a liquid nitrogen storage bank and transfer them on dry ice to a tissue culture room. Thaw frozen vials of cells by gently swirling them in the 37 °C water bath until a small pellet of ice remains in the cryovial (*see* **Note 10**).
4. Transfer the vials to a laminar flow biosafety cabinet. Using a 2 mL serological pipette, quickly but carefully add 1 mL of warm wash medium dropwise to thawed cells and transfer the cell suspension to a 15 mL centrifuge tube with the remaining wash medium. Using the same pipette, wash the inside of the cryovial once with 1 mL of wash medium. Carefully mix the cell suspension in the 15 mL tube with a light swirling motion and by gently tapping the tube with a finger. Set aside a small aliquot of the cell suspension to evaluate cell viability using trypan blue and count cells.
5. Centrifuge cell suspension in 15 mL tubes at 150 g for 3 minutes. In the meantime, particularly if transplanting LGE progenitors, evaluate cell viability and count cells in the cell suspension that was set aside in the previous step. If

differentiating LGE progenitors into MSNs *in vitro*, also use this time to transfer the culture plates from the incubator to the microbiological safety cabinet.

6. After centrifugation is completed, aspirate the supernatant from the centrifuge tube with a vacuum pump without disturbing the cell pellet. Disrupt the cell pellet by gently flicking the tube.
7. If LGE progenitors are thawed for transplantation into an animal model of HD, resuspend cells in DMEM/F-12 at the required concentration (e.g. 1–2.5 x 10^5 cells/µl) and proceed with transplantation experiments. If thawed cells are going to be differentiated into MSNs *in vitro*, resuspend cells in the appropriate volume of LGE patterning medium to seed 0.5–1 x 10^6 cells/cm^2 in 1 mL/well for 12- and 24-well plates.
8. Seed LGE progenitors into the culture container and transfer the plate to a humidified 5% CO_2 37 °C incubator and ensure cells are distributed evenly across wells by gently moving the plate in several quick, short, side-to-side and back-and-forth motions. On the next day, proceed to Step 5 in ***3.5***.

4 Notes

1. If hPSCs are maintained on feeders, collect hPSCs and pre-plate cultures on gelatin for 45 minutes to remove feeder cells before proceeding to seeding cells for a differentiation.
2. We observed a high batch to batch variability with CELLstart when using it as a matrix for differentiation. With some batches of CELLstart forebrain progenitors detach from the matrix after 9 days *in vitro*.
3. The majority of hPSC lines can by maintained in mTeSR™1 or E8 medium culture media on Matrigel matrix or in StemPro medium on Matrigel, CELLstart (1:50 in DBPS) or Laminin + Fibronectin matrix (10 µg/mL + 5 µg/mL in DPBS,

respectively). However, RC lines (e.g. RC9 and RC17) are best maintained in StemPro culture medium on any of the three matrices.

4. For a successful outcome, hPSC cultures must comprise of homogeneously distributed colonies of healthy pluripotent cells, with no contaminating differentiated cells. All differentiated colonies should be removed before the start of the experiment. When working with a new hPSC line, it is good practice to assess cell pluripotency using IF staining or qRT-PCR before the start of the neural induction procedure.
5. CELLstart (1:50 in DBPS) or Laminin + Fibronectin matrices (10 µg/mL + 5 µg/mL in DPBS, respectively) can also be used at this stage without any effect on the efficiency of LGE fate induction.
6. Do not triturate hPSCs to a single cell suspension, as this severely compromises hPSC survival. If the exact number of cells is required for plating, one 70 – 80% confluent well of a 6-well plate may be sacrificed and treated with Accutase Cell Dissociation Agent for 10 minutes at 37 °C to prepare a single cell suspension for cell counting using a vital staining, such as trypan blue.
7. For a successful neuronal differentiation, seeded cells must be in a monolayer with no cell aggregates present. It is equally important that cell colonies are evenly distributed across the well to ensure homogeneous cell growth and subsequent neural induction. Denser areas of cells will expand quicker and form multilayer structures introducing heterogeneity. If cells seeded on GFR Matrigel have attached as aggregates, it could be due to (i) too prolonged incubation time during coating (do not exceed 1.5 hours) or (ii) coating solution drying out. Use sufficient volume of coating solution to avoid it drying out.
8. While the most convenient stage for this passage is at the end of neural induction phase at D9, depending on the speed of neural induction for a given

hPSC line/culture, cells may have to passaged before D9. In our hands, forebrain progenitors have been successfully transferred to Fibronectin-coated culture vessels during D7-12 of differentiation. However, extra care needs to be taken to be very gentle with cells if passaging cultures earlier than D9. Although patterning of forebrain progenitors towards an LGE fate can be initiated on D9 of differentiation, we prefer to delay this by one day if cells are being passaged on D9. This allows time for the neural progenitors to recover from passaging and avoid potential waste of activin should the progenitors fail to survive and adhere well.

9. Do not incubate forebrain progenitor cultures in EDTA for longer than 2 minutes as it would result in too much dissociation and lead to low cell survival after passaging. At this stage, neuronal precursors are highly sensitive to the size of cell clusters they are passaged in. Do not triturate and dissociate cell suspension to small clumps (<300 μm), as this severely compromises forebrain progenitor survival.
10. When thawing cells, do not keep cryovials in the water bath until all ice melts, especially if LGE progenitors were frozen in 10% (vol/vol) DMSO solution in the LGE patterning medium. Do not allow thawed cells to dwell in the freezing medium, as prolonged exposure to DMSO severely affects cell survival. Transfer thawed cells to the wash medium as quickly as possible to dilute DMSO. Minimise manipulation of the cell suspension with pipettes as newly thawed LGE progenitors are delicate.

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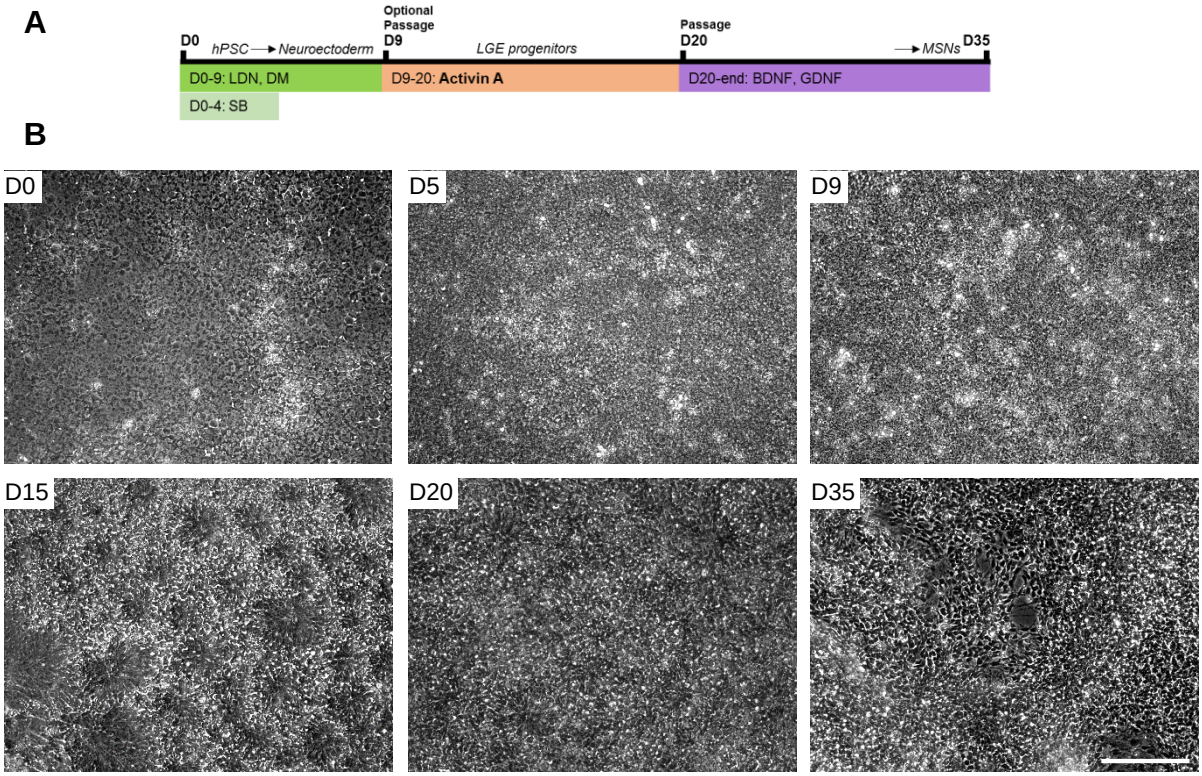

Figure 1. Representative images illustrating the expected cell morphology along the protocol. (A) Experimental scheme. (B) Bright field images with phase-contrast showing characteristic hES H9 cell morphology at different stages along the MSN differentiation timeline. Scale bar: 200 µm in B.

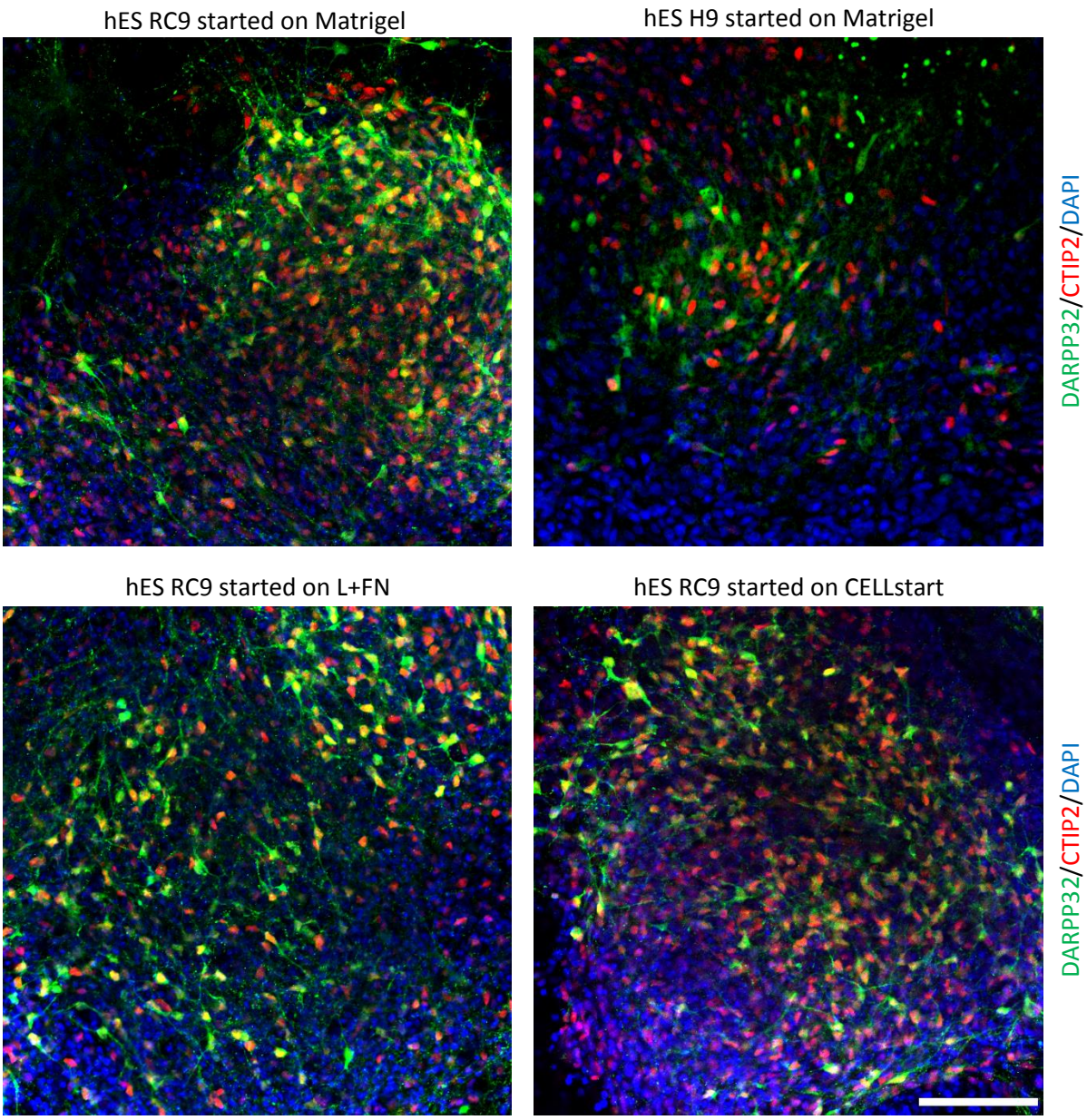

Figure 2. Efficient generation of medium spiny neurons by activin stimulation. Immunostaining for DARPP32 and CTIP2 of D43–45 neurons derived from hES H9 and RC9 cells in different culture systems. Scale bar: 100 μm for all panels.

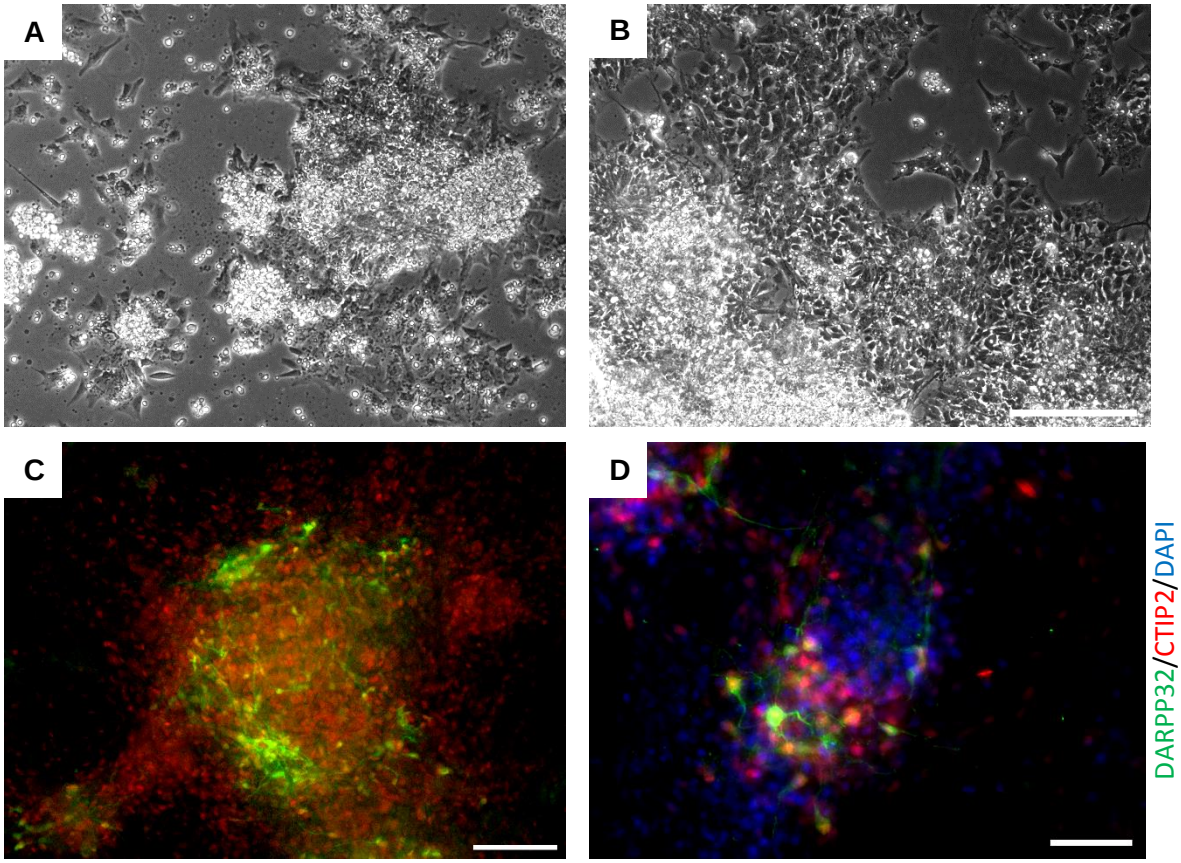

Figure 3. Generation of striatal projection neurons from cryopreserved LGE progenitors. (A) Bright field image with phase-contrast of forebrain progenitors, cryopreserved at D9, next day after thawing illustrating very low survival of telencephalic precursor cells. (B) Bright field image with phase-contrast of LGE progenitors, cryopreserved at D20, next day after thawing showing good survival and recovery of neural progenitors with some rosette formation. (C, D) Immunostaining of D33 cultures for the indicated markers demonstrating a retained potential of LGE progenitors to generate MSNs after cryopreservation process. Scale bars: 200 µm in A-C, 50 µm in D.

TABLE 1: Antibodies used for immunofluorescence

Antibody	Supplier	Cat. No.	Dilution
Anti-Calbindin	Swant	CB-38a	1:500
Anti-Ctip2	Abcam	25B6	1:500
Anti-DARPP32	Santa Cruz	Sc-11365	1:200
Anti-DLX2	Millipore	Ab5726	1:300
Anti-ENK	Immunostar	20065	1:400
Anti-SubP	Immunostar	20064	1:400
Anti-FOXP1	Abcam	Ab16645	1:500
Anti-FOXP2	Abcam	Ab58599	1:100
Anti-FOXG1	Abcam	Ab5274	1:250
Anti-GABA	Sigma	A2052	1:500
Anti-GAD65/67	Sigma	G5163	1:1000
Anti-GSX2	Millipore	Abn162	1:1000
Anti-HuNu	Millipore	Mab1281	1:250
Anti-hNCAM	Santa Cruz	Sc-106	1:200
Anti-MAP2	Sigma	M1406	1:250
Anti-Nestin	BD	611659	1:300
Anti-NeuN	Millipore	Mab377	1:250
Anti-NKX2.1	Abcam	Ab40880	1:1000
Anti-NOLZ1	Abnova	H00084858-B01F	1:500
Anti-OTX2	Millipore	Ab9566	1:300
Anti-PAX6	DSHB	Ab528427	1:1000
Anti-PSD95	Thermo Scientific	6G6-1C9	1:200
Anti-TH	Pelfreez	P40101	1:500
Alexa Fluor®	Life Technologies	Multiple	1:200

TABLE 2: List of qPCR primers

Gene Name	Forward primer sequence	Reverse primer sequence
ARPP21	GGAAGCTGGTTGACGATGTGTC	GGCTTCTGTCGTTCTACGCC
βActin	TCACCACCACGGCCGAGCG	TCTCCTTCTGCATCCTGTCG
Calbindin	ATCAGGACGGCAATGGATAC	TAAGAGCAAGATCCGTTCGG
CTIP2	CTCCGAGCTCAGGAAAGTGTC	TCATCTTTACCTGCAATGTTCTCC
DARPP32	TTGGAAAATCCAGAAAACCG	CTGGTAGAAGCCGGTGAGAG
DLX2	ACTACCCCTGGTACCACCAGAC	TCTGCTCTCAGTCTCTGGCGAGTTCTC
DRD2	CTGAGGGCTCCACTAAAGGAG	CATTCTTCTCTGGTTTGGCG
EBF1	AATGTAAGCAAGGTGGACGC	TCAAGGTCTAAGCCGGACAC
FOXG1	TGGCCCATGTCGCCCTTCCT	TGGCCCATGTCGCCCTTCCT
FOXP2	AATGTGGGAGCCATACGAAG	GCCTGCCTTATGAGAGTTGC
GAD67	CGTCTTCGACCCCATCTTCGT	CGCAGATCTTGAGCCCCAGTT
GAPDH	ATGACATCAAGAAGGTGGTG	CATACCAGGAAATGAGCTTG
GLI1	TGAGGCCCTTCAAAGCCC	GTATGACTTCCGGCACCCTTC
GSX2	TCACTAGCACGCAACTCCTG	TTTTCACCTGCTTCTCCGAC
NKX2.1	CGCATCCAATCTCAAGGAAT	TGTGCCCAGAGTGAAGTTTG
NOLZ1	ACATTTTGCACCCCGAGTAC	GGAGTACGGCTTGAAACTCG
PAX6	AACAGACACAGCCCTCACAAACA	CGGGAACTTGAACTGGAACTGAC
PENK	GCTGTCCAAACCAGAGCTTC	TCTGGCTCCATGGGATAAAG
PTCH1	TTCGCTCTGGAGCAGATTTCCAAG	GCTTTTAATCCCACCGCGAAG
TAC1	TGGGGTTGAAAATTCAAAAAG	GGAGTTTCCTTCCTTTTCCG
TH	GAGTACACCGCCGAGGAGATTG	GCGGATATACTGGGTGCACTGG
vGLUT1	AGTTCGCAACGATGATGGCA	CTGCACCCCAGCATCTCTGA