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TITLE:
Efficient and Scalable Generation of Human Ventral Midbrain Astrocytes from Human-Induced Pluripotent Stem Cells

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KEYWORDS:
astrocyte, Parkinson's disease, ventral midbrain, neuroinflammation, reactive human induced pluripotent stem cell, differentiation

SUMMARY:
Here, we present a method for reproducible generation of ventral midbrain patterned astrocytes from hiPSCs and protocols for their characterization to assess phenotype and function.

ABSTRACT:
In Parkinson's disease, progressive dysfunction and degeneration of dopamine neurons in the ventral midbrain cause life-changing symptoms. Neuronal degeneration has diverse causes in Parkinson's, including non-cell autonomous mechanisms mediated by astrocytes. Throughout the CNS, astrocytes are essential for neuronal survival and function, as they maintain metabolic homeostasis in the neural environment. Astrocytes interact with the immune cells of the CNS, microglia, to modulate neuroinflammation, which is observed from the earliest stages of Parkinson's, and has a direct impact on the progression of its pathology. In diseases with a chronic neuroinflammatory element, including Parkinson's, astrocytes acquire a neurotoxic phenotype, and thus enhance neurodegeneration. Consequently, astrocytes are a potential therapeutic target to slow or halt disease, but this will require a deeper understanding of their properties and roles in Parkinson's. Accurate models of human ventral midbrain astrocytes for in vitro study are

therefore urgently required.

We have developed a protocol to generate high purity cultures of ventral midbrain-specific astrocytes (vmAstros) from hiPSCs that can be used for Parkinson's research. vmAstros can be routinely produced from multiple hiPSC lines, and express specific astrocytic and ventral midbrain markers. This protocol is scalable, and thus suitable for high-throughput applications, including for drug screening. Crucially, the hiPSC derived-vmAstros demonstrate immunomodulatory characteristics typical of their in vivo counterparts, enabling mechanistic studies of neuroinflammatory signaling in Parkinson's.

INTRODUCTION:

Parkinson's disease affects 2%–3% of people over 65 years of age, making it the most prevalent neurodegenerative movement disorder[1]. It is caused by degeneration of ventral midbrain dopamine neurons within the substantia nigra, resulting in debilitating motor symptoms, as well as frequent cognitive and psychiatric issues[2]. Parkinson's pathology is typified by aggregates of the protein, α-synuclein, which are toxic to neurons and result in their dysfunction and death[1–3]. As the dopaminergic neurons are the degenerating population in Parkinson's, they were historically the focus of research. However, it is apparent that another cell type in the brain, the astrocytes, also demonstrate abnormalities in Parkinson's, and are believed to contribute to degeneration in models of Parkinson's[4–7].

Astrocytes are a heterogenous cell population that can transform both physically and functionally as required. They support neuronal function and health via a plethora of mechanisms, including the modulation of neuronal signaling, shaping of synaptic architecture, and trophic support of neuronal populations via secretion of specific factors[6,8–10]. However, astrocytes also have a substantial immunomodulatory role, integral to the development and propagation of neuroinflammation[10,11]. Neuroinflammation is observed in the affected brains, and significantly has recently been shown to pre-empt the onset of Parkinson's symptoms[12–15], thereby taking the center stage in Parkinson's research.

At a cellular level, astrocytes are said to become reactive in response to injury, infection, or disease, as an attempt to facilitate neuroprotection[9,6,10,16]. Reactivity describes a shift in astrocyte phenotype characterized by changes in gene expression, secretome, morphology, and mechanisms of clearance of cell debris and toxic byproducts[9–11,17]. This reactive shift occurs in response to inductive signals from microglia, which are the immune cells of the CNS and the first responders to injury and disease[9]. Both astrocytes and microglia respond to inflammatory signals by moderating their own function and can transduce inflammatory signals and thus directly influence neuroinflammation[9,10]. However, the chronic nature of Parkinson's results in a transition where reactive astrocytes become toxic to neurons, and themselves promote degeneration and disease pathology[6,9,10,18,19]. Significantly it was recently demonstrated that blocking the transformation of astrocytes into the reactive neurotoxic phenotype prevents the progression of Parkinson's in animal models[11]. Astrocyte reactivity in the paradigm of neuroinflammation has therefore become a major focus of Parkinson's research, and similarly relates to a wide spectrum of diseases of the CNS. Together these findings build a picture of

significant astrocytic involvement in the etiology of Parkinson's, emphasizing the need for accurate research models that recapitulate the phenotype of the human astrocyte populations that are involved in Parkinson's.

In the embryonic brain, neurons appear first, with the astroglial lineage, namely, the astrocytes and oligodendrocytes, appearing later in development[6]. In vivo and in vitro studies have highlighted a number of signaling pathways that appear to control the potency of neural progenitor cells from neuronal to astroglial derivatives. In particular, JAK/STAT, EGF, and BMP signaling play roles in the proliferation, differentiation, and maturation of astroglia[20,21]. These pathways have been the focus of in vitro protocols for the generation of astrocytes from pluripotent cells, including hiPSC[6,22,23]. There have been many successful examples of generating astrocytes from hiPSCs[6,24,25]. However, it is apparent that in vivo astrocytes in the CNS possess specific regional identities, which relate directly to their function, in accordance with the specific requirements of those astrocytes in relation to their specialized neuronal neighbors[17,24–26]. For example, relating specifically to the ventral midbrain, it has been demonstrated that astrocytes in this region express specific sets of proteins, including receptors for dopamine enabling communication with the local population of midbrain dopamine neurons[26]. Furthermore, ventral midbrain astrocytes demonstrate unique signaling properties[26]. Therefore, to study the role of ventral midbrain astrocytes in Parkinson's, we require an in vitro model that reflects their unique set of characteristics.

To address this, we have developed a protocol to generate ventral midbrain astrocytes (vmAstros) from hiPSCs. The resulting vmAstros exhibit characteristics of their in vivo ventral midbrain counterparts such as expression of specific proteins, as well as immunomodulatory functions. The results presented are from the differentiation of the NAS2 and AST23 hiPSC lines, which were derived and gifted to us by Dr. Tilo Kunath[27]. NAS2 was generated from a healthy control subject whereas AST23 is derived from a Parkinson's patient carrying a triplication in the locus encoding α-Synuclein (SNCA). These hiPSC lines have been previously characterized and used in a number of published research papers, including for the generation of various neural cell types[27–31].

PROTOCOL:

1. Human hiPSC line thawing, maintenance, and cryopreservation

1.1. For coating hiPSC culture plates, dilute vitronectin to 5 µg/mL (1:100) in PBS at 1 mL per 10 cm^2 cell culture plate surface area. Leave for 1 h at room temperature.

1.2. Remove vitronectin and proceed immediately to adding hiPSCs/media to the culture plate.

NOTE**:** When removing vitronectin from the plate, it is crucial that the culture surface is not allowed to dry out.

1.3. To thaw hiPSCs Remove cryovials containing hiPSCs from liquid nitrogen and place in a 37 °C water bath until the contents have completely thawed.

1.4. Prepare 9 mL of prewarmed cell culture medium (e.g., E8 or E8 Flex) containing 1x cell supplement (e.g., Revitacell). Add 1 mL dropwise to the contents of the cryovial. Place the remaining 8 mL media into a 15 mL centrifuge tube and to this add the diluted contents of the cryovial.

CAUTION: Do not triturate the contents.

1.5. Centrifuge at 150 x *g* for 3 min. Aspirate the liquid without disturbing the cell pellet and resuspended in an appropriate volume of cell culture medium (e.g., E8 or E8 Flex) containing 1x cell supplement (e.g., Revitacell). For example, 2 mL per well of a 6-well plate.

1.6. Add resuspended hiPSCs to vitronectin coated dishes and place in 37 °C/5% CO_2 incubator.

NOTE: hiPSCs should start to attach to vitronectin coated plasticware in 30 min–2 h after thawing.

1.7. Maintain hiPSCs in cell culture medium (e.g., E8 or E8 Flex). Feed cells daily by media exchange. Always prewarm culture media for 30 min before feeding.

NOTE**:** If using E8 Flex, hiPSCs do not require media changes every 24 h and feeding increments can be extended to 48 h, if needed. Either E8 Flex or E8 media yield equally high-quality hiPSC cultures. HiPSCs should be cultured for a minimum 14 days post-thawing, and prior to beginning the differentiation steps. Culture periods of less than 14 days appear to negatively impact the survival of the hiPSCs during the initial differentiation period.

NOTE: Passage hiPSCs at approximately 80% confluency (**Figure 1A**: 3–4 day passaging interval).

1.8. 1 h prior to beginning, add 1x cell supplement (e.g., Revitacell) to the hiPSC culture.

1.9. Wash hiPSCs once with PBS (without calcium or magnesium) and add 0.5 mM EDTA (diluted from stock in PBS without calcium or magnesium).

1.10. Incubate for 5 min at room temperature, or until the hiPSCs begin to detach from each other and take on a more rounded appearance, with the boundaries of each iPSC appearing brighter under a brightfield microscope.

1.11. Add 200 µL EDTA on to a focused area of the hiPSCs with a pipette. If they readily detach, making a clear space in the cell layer, then they are ready to be harvested. If they do not readily detach, leave in EDTA and repeat after 1 min.

1.12. When ready to proceed, gently remove EDTA, and using a pipette, gently wash the hiPSCs twice with cell culture medium (e.g., E8 or E8 Flex).

174 NOTE: To achieve this without the hiPSCs detaching, tip the plate and add media dropwise down
175 the side of the culture plate.
176
177 1.13. To harvest hiPSCs use 1 mL cell culture medium (e.g., E8 or E8 Flex) containing 1x cell
178 supplement (e.g., Revitacell). Release the media directly onto the hiPSC layer and the cells
179 should detach. If required, repeat with another 1 mL media.
180
181 1.14. View the hiPSCs under the microscope. Ideally, hiPSCs should appear in relatively uniform
182 clusters as shown in **Figure 1B**. If hiPSC clusters are much larger, or very variable in size, use
183 the pipette to break up the larger hiPSC clusters (**Figure 1B**).
184
185 NOTE**:** Do not over-triturate hiPSCs. Although the supplement increases the overall cell survival,
186 over trituration negatively impacts on the survival of the hiPSC culture. 1–4 passes with a pipette
187 are recommended.
188
189 1.15. Using a serological pipette, transfer the hiPSC suspension on to a vitronectin-coated plate
190 as prepared in **step 1.1**. Return the hiPSC culture to the 37 °C/5% CO_2 incubator.
191
192 NOTE: Cryopreserve hiPSCs at approximately 80% confluency.
193
194 1.16. 1 h prior to beginning, add 1x cell supplement (e.g., Revitacell) to the hiPSC culture.
195
196 1.17. Detach hiPSCs from culture plates using 0.5 mM EDTA as described in **step 1.4**, collecting
197 cells in cell culture medium (e.g., E8 or E8 Flex) containing 1x cell supplement (e.g.,
198 Revitacell). Centrifuge at 150 x *g* for 3 min.
199
200 1.18. Resuspend pelleted hiPSCs in cell freezing media (see **Table of Materials**). Use 700 µL per
201 10 cm^2 culture area, equivalent to 1 cryovial of cells per well of a 6-well plate.
202
203 1.19. Transfer cryovials into an appropriate cell freezing vessel (for details see **Table of**
204 **Materials**).
205
206 1.20. Transfer the freezing vessel to a -80 °C freezer for 24 h. After 24 h, cryovials can be
207 transferred to liquid nitrogen (-196 °C) for long-term storage.
208
209
210 **2. <u>vmAstro Differentiation protocol</u>**
211
212 NOTE: A schematic summary of the vmAstros differentiation protocol is shown in **Figure 1A**. A
213 detailed list of reagents required for the protocol and their preparation is given in **Table 1**.
214
215 **2.1. Induction of vmNPCs**
216
217 NOTE: This protocol has been optimized to begin with a minimum with 1x well of a 6-well plate

(10 cm^2) of hiPSCs 70%–80% confluency, which is approximately 4–5 x 10^4 cells/cm^2 (**Figure 1B**)[30]. Starting cell number and density must be optimized for each hiPSC line as it significantly impacts survival and differentiation efficiency.

2.1.1. Remove cell culture medium from hiPSCs and wash 3x in DMEM/F12 + glutamax. Replace media with 2 mL vmNPC induction media (N2B27 + CHIR99021 + SB431542 + SHH(C24ii) + LDN193189. See **Table 1** for details of preparing media and reagents).

2.1.2. Feed on alternate days with a half media change after 24 h, and a full media change at 48 h.

NOTE: After 3–4 days the vmNPC culture will require passaging. A standard passaging ratio of 1:3 or 1:4 is recommended—this needs to be optimized for each hiPSC line used.

2.1.3. 1 h before passaging, add 1x cell supplement (e.g., Revitacell) to vmNPCs, and prepare 1x basement membrane matrix (e.g., Geltrex) coated tissue culture plastic (see **section 2.2 'Preparing basement membrane matrix and coating tissue culture plastic'**).

2.1.4. Remove the media from vmNPCs and wash 2x with D-PBS. Add 1 mL pre-warmed cell detachment solution (e.g., Accutase) per 10 cm^2 culture area (1 mL per well of a 6-well plate).

2.1.5. Place at 37 °C for 1 min and then examine vmNPCs using a phase contrast microscope.

NOTE: The vmNPCs will start to round up, their processes will re-tract, and gaps will appear in the cell layer. This can take from 1–3 min depending on cell density.

2.1.6. When vmNPCs take on this appearance, add 100 µL of cell detachment solution (e.g., Accutase) on to the layer of vmNPCs.

NOTE: If the vmNPCs are ready to detach, a hole in the cell layer will appear. If this doesn't happen, then the vmNPCs require further incubation with cell detachment solution.

2.1.7. If vmNPCs readily detach, then gently remove the cell detachment solution and wash vmNPCs 2x with N2B27 media. Add N2B27 media gently down the side of the well or culture vessel and gently swirl to wash, ensuring that vmNPCs do not detach.

NOTE: This step must be completed quickly to ensure vmNPCs do not reattach to the cell surface. If vmNPCs start to detach in the wash steps, collect via centrifugation at 150 x *g* for 3 min. vmNPCs are not centrifuged as standard when passaging as this can reduce their survival.

2.1.8. Finally, remove vmNPCs using a pipette, by vigorously ejecting vmNPC induction media containing 1x cell supplement (e.g., Revitacell) directly on to the cell layer. This should remove vmNPCs, which can then be transferred directly into the new matrix-coated coated culture vessel.

NOTE: Do not re-use media already containing resuspended vmNPCs to remove further cells as this will result in their over-trituration, which reduces their survival.

2.1.9. Replace in 37 °C/5% CO_2 incubator. vmNPCs should begin to attach to the matrix-coated surface after 20–30 min. Replace half of the media with fresh vmNPC induction media (without cell supplement) after 24 h and continue the feeding schedule as earlier.

2.1.10. Continue the regime of feeding and passaging for 10 days.

2.2. Preparing basement membrane matrix and coating tissue culture plastic

NOTE: For maintaining vmNPCs 1x basement membrane matrix (e.g., Geltrex) is used for coating plasticware. For maintaining vmAPCs or vmAstros, 0.25x basement membrane matrix can be used.

2.2.1. Remove basement membrane matrix stock from a -80 °C freezer and place in a 4 °C fridge overnight to thaw.

2.2.2. Dilute 1:10 with ice cold DMEM/F12 + glutamax, aliquot and store at -80 °C as a 10x stock.

2.2.3. When coating plasticware dilute this 10x stock to 1x (for vmNPCs) or 0.25x (for vmAPCs or vmAstros) with ice cold DMEM/F12 + glutamax.

2.2.4. Immediately add to tissue culture plastic at 1 mL per 10 cm^2, for example, 1 mL per well of a 6-well plate.

2.2.5. Place at 37 °C for 1 h. The basement membrane matrix solution should not be removed from plasticware until ready to add media/cells to ensure the coated plasticware does not dry out. Matrix coated plates do not require washing before adding cells.

2.3. Expansion of vmNPCs

2.3.1. On day 10 of the protocol, replace the induction media with vmNPC expansion media (N2B27 + GDNF + BDNF + ascorbic acid. See **Table 1** for details of preparing media and reagents).

NOTE**:** The vmNPCs do not require the addition of mitogens to induce proliferation. BDNF, GDNF, and ascorbic acid support the survival and maintenance of vmNPCs[30].

2.3.2. Feed on alternate days with a half media change after 24 h, and a full media change at 48 h.

306
307 NOTE: After 3–4 days, the vmNPC culture will require passaging. For passaging, a standard
308 passaging ratio of 1:3 or 1:4 is recommended (this needs to be optimized for each hiPSC line used.
309 Determine the ratio that gives the best survival, proliferation, and generation of vmNPCs).
310
311 2.3.3. 1 h before passaging, add 1x cell supplement (e.g., Revitacell) to vmNPCs, and prepare 1x
312 matrix coated plates/flasks in advance (see **section 2.2 'Preparing basement membrane matrix
313 and coating tissue culture plastic'**). Prewarm the cell detachment solution (e.g., Accutase) to 37
314 °C. Prewarm fresh vmNPC expansion media containing 1x cell supplement (e.g., Revitacell).
315
316 2.3.4. Remove media from vmNPCs and wash 2x with D-PBS. Add 1 mL cell detachment solution
317 (e.g., Accutase) per 10 cm^2 culture area (1 mL per well of a 6-well plate). Place at 37 °C for 1 min
318 and then examine vmNPCs using a phase-contrast microscope.
319
320 NOTE: The vmNPCs will start to round up, their processes will re-tract, and gaps will appear in
321 the cell layer. This can take from 1–3 min depending on the cell density.
322
323 2.3.5. When vmNPCs takes on a rounded appearance, add 100 µL of cell detachment solution
324 (e.g., Accutase) on to the layer of vmNPCs.
325
326 NOTE: If the vmNPCs are ready to detach, a hole in the cell layer will appear. If this doesn't
327 happen, then the vmNPCs require further incubation with the cell detachment solution.
328
329 2.3.6. If vmNPCs do readily detach, then gently remove the cell detachment solution and wash
330 vmNPCs twice with N2B27 media. Add the N2B27 media gently down the side of the well or
331 culture vessel and gently swirl to wash, ensuring that vmNPCs do not detach.
332
333 NOTE: If vmNPCs start to detach in the wash steps, collect via centrifugation at 150 x *g* for 3 min.
334 vmNPCs are not centrifuged as standard when passaging as this can reduce their survival. This
335 step must be completed quickly to ensure vmNPCs do not reattach to the cell surface.
336
337 2.3.7. Remove vmNPCs using a pipette, and vigorously eject vmNPC expansion media containing
338 1x cell supplement (e.g., Revitacell) directly on to the cell layer. This should remove vmNPCs,
339 which can then be transferred to a 15 mL centrifuge tube.
340
341 NOTE: Do not re-use media already containing resuspended vmNPCs to remove further cells as
342 this will result in their over-trituration, which reduces their survival.
343
344 2.3.8. Replace in 37 °C/5% CO_2 incubator. vmNPCs should begin to attach to the matrix coated
345 surface after 20–30 min. After 24 h, replace half of the culture media with fresh vmNPC expansion
346 media (without cell supplement) and continue the previous feeding schedule.
347
348 2.3.9. Continue this regime of feeding and passaging for 10 days. vmNPCs can be expanded up
349 to day 50.

2.4. Differentiation and expansion of vmAPCs

NOTE: vmNPCs can be used successfully for the generation of vmAPCs/vmAstros anywhere between 30 and 50 days from the initial hiPSC stage (**Figure 1A**).

2.4.1. Take a confluent vmNPC culture and wash vmNPCs 3x in advanced DMEM/F12 to remove traces of the components of vmNPC expansion media. Replace the media with vmAPC expansion media (ASTRO media +EGF +LIF. See **Table 1** for details of preparing media and reagents).

2.4.2. After 72 h passage, the vmNPC culture is at a high ratio (1:7.5). For example, assuming vmNPCs were maintained in a single well of a 6-well plate, they should now be passaged into a 1x matrix coated 75 cm^2 flask (coated as described in **section 2.2 'Preparing basement membrane matrix and coating tissue culture plastic'**). Passage using cell detachment solution, as described for vmNPCs in **section 2.3** '**Expansion of vmNPCs'.**

2.4.3. Resuspend vmNPCs in an appropriate volume of vmAPC Expansion media (7.5–15 mL media per 75 cm^2 flask. Complete media changes every 3 days, or as the cells require.

NOTE: From this point on, vmNPCs are referred to as vmAPCs and should be passaged as single cells rather than cell clusters. vmAPCs should be passaged every 3–7 days or as they become confluent to avoid becoming over confluent. From this point onward the reduced concentration of 0.25x matrix should be used to coat plasticware (as described in **section 2.2 'Preparing basement membrane matrix and coating tissue culture plastic'**).

2.4.4. Expand vmAPCs until they reach day 90 (from the hiPSC stage), cryopreserving vmAPCs at various points in their expansion.

2.5. Generation of mature vmAstros from vmAPCs

NOTE: At this stage, vmAPCs can be grown in 175 cm^2 tissue culture flasks. This may be expanded for the generation of large numbers of mature vmAstros.

2.5.1. When vmAPCs reach 80% confluency, wash 3x with ASTRO media and replace with vmAstros maturation media (ASTRO media +BMP4 +LIF. See **Table 1** for details of preparing media and reagents).

2.5.2. Carry out a complete media change every 3 days, or as the cells require, for 10 days.

NOTE: a) At this point, characterization indicates that the vmAstros are mature, as confirmed by immunocytochemistry (**Figure 2G–I**) and by gene expression analysis (manuscript in preparation). b) vmAstros used immediately after maturation should be re-plated on a newly prepared matrix-

coated surface. Maintaining either vmAPCs or vmAstros on the same culture surface for over 14 days could lead to suboptimal cultures, where cells begin to shrink in size and even detach. c) For applications examining neuroinflammatory modulation, BMP and LIF are removed 72h prior to neuroinflammatory stimulation. This is to avoid any potential interaction between BMP/LIF signalling and induced neuroinflammatory signalling.

2.5.3. vmAstros can now be re-plated for experimental assays, for example, onto coverslips for immunocytochemistry or cryopreserved for future applications.

NOTE: Passaging should not be necessary at this stage of the protocol as proliferation should only occur at a very low rate. vmAPCs plated too densely at this stage maintain higher levels of proliferation. If this is the case, passage and split cells to achieve a density as is shown in **Figure 1F**.

3. Cryopreservation of vmNPCs, vmAPCs, and vmAstros

NOTE: Cryopreserve vmNPCs/vmAPCs/vmAstros at full confluency.

3.1. 1 h prior to beginning, add 1x cell supplement (e.g., Revitacell) to culture. Fill the cryostorage vessel (see **Table of Materials**) with isopropanol at room temperature.

3.2. Detach the cells from the culture plates using cell detachment solution as previously described, collecting cells in appropriate media (N2B27 or ASTRO media) containing 1x cell supplement (e.g., Revitacell). Centrifuge at 150 x *g* for 3 min.

3.3. Resuspend pelleted vmNPCs/vmAPCs/vmAstros in cell freezing media (see **Table of Materials**) volumes as follows:

- vmNPCs: 700 µL per 10 cm^2 culture area, into 1 cryovial.
- vmAPCs: 700 µL per 60 cm^2 culture area, into 1 cryovial (approximately 1/3 of a T175 culture flask).
- vmAstros: Resuspend in a 2 mL of media and count the number of vmAstros. Re-centrifuge and resuspend in cell freezing media (see **Table of Materials**) at a number per cryovial appropriate to future applications. Assuming an approximate cell loss of 15% due to freeze-thawing, newly thawed vmAstros are counted and plated with a 15% excess cell number to compensate for cell death in the freeze-thaw process. This is, therefore, equivalent to 74,750 vmAstros per cm^2. Thawed vmAstros are maintained for 72 h in ASTRO media prior to assaying.

3.4. Transfer the cryovials into a cell freezing vessel (for details see **Table of Materials**) and transfer the freezing vessel to a -80 °C freezer for 24 h. After 24 h, cryovials can be transferred to liquid nitrogen (-196 °C) for long-term storage.

4. Characterization of vmAstro phenotype

4.1. Immunocytochemistry

4.1.1. Place 100–200 13 mm glass coverslips in glass Petri dishes on a layer of filter paper and sterilize them in a dry autoclave.

4.1.2. Transfer the coverslips to wells of 4- or 24-well plates using sterile forceps. Add 1x matrix solution (e.g., Geltrex) on the coverslips as 50 µL droplets and incubate at 37 °C for 1 h.

4.1.3. Passage or thaw vmAstros, resuspend in ASTRO media and carry out a count. Plate vmAstros at 25–100,000 cells per coverslip in a 50 µL droplet.

4.1.4. Remove the matrix from the coverslip and immediately add vmAstros in a droplet of media. Place at 37 °C for 30 min and then flood the wells with an additional 250 µL ASTRO media.

NOTE: If carrying out immunocytochemistry to simply check for astrocyte and midbrain marker expression, vmAstros can be fixed 24 h after plating.

4.1.5. Prepare 4% formaldehyde solution by diluting 36% formaldehyde solution 1:9 in D-PBS.

4.1.6. Wash vmAstros 1x with D-PBS. Immediately add 4% formaldehyde to the wells and leave at room temperature for 10 min.

4.1.7. Remove formaldehyde and replace with D-PBS. Either store at 4 °C or proceed to immunocytochemistry.

4.1.8. Wash coverslips in wells 3x with D-PBS. Permeabilize and block in 10% goat serum, 1% BSA in 0.1% PBTx (D-PBS + 1:1000 Triton-X) for 1 h at room temperature.

4.1.9. Add primary antibodies (**Table of Materials**) in 1% goat serum, 0.1% BSA in 0.1% PBTx (D-PBS + 1:1000 Triton-X) and incubate overnight at 4 °C on a rocker.

4.1.10. On the next day, remove primary antibodies and wash coverslips 3x with D-PBS.

4.1.11. Add appropriate secondary antibodies (**Table of Materials**) in 1% goat serum, 0.1% BSA in 0.1% PBTx, and incubate for 1–2 h at room temperature and protect it from light on a rocker. Wash coverslips 3x with D-PBS.

4.1.12. Add DAPI solution (0.1 µg/mL DAPI in D-PBS) and incubate at room temperature for 10 min. Wash coverslips 3x with D-PBS.

481 **4.1.13.** To mount the coverslip, add a 5 μL droplet of Mowiol/DABCO mounting media [12% Mowiol (w/v), 12% glycerol (w/v) dissolved overnight stirring in 0.2 M Tris (pH 8.5) with 25 mg/mL 1,4-diazabicylo[2.2.2]octane (DABCO)] to a glass microscope slide. Using forceps, carefully remove the coverslip from the well; dab the edge of the coverslip on the tissue to remove the excess liquid and place vmAstros side down onto the Mowiol/DABCO droplet.

4.1.14. Repeat for each coverslip and leave to dry for 8 h before microscopic examination.

4.2. ELISA measurement of vmAstro secreted IL-6 in response to cytokine treatment

NOTE: Following the 10-day maturation with BMP4 and LIF, vmAstros should be passaged, counted, and plated on 0.25x matrix-coated tissue culture plasticware (as described in **section 2.2 'Preparing basement membrane matrix and coating tissue culture plastic'**), at a density of 65,000 vmAstros per cm^2 in ASTRO media. BMP4 and LIF should be removed from the vmAstros 72 h prior to cytokine treatment as active signaling from these factors can interfere with the efficacy of the cytokine treatment. Alternatively, cryopreserved vmAstros can be thawed and used for assays.

4.2.1. On the day of the assay, gently wash vmAstros 3x in non-redox media (DMEM/F-12 + glutamax + N2).

4.2.2. Use an untreated control and cytokine-treated well for comparison. Add chosen cytokine at optimized concentration. The data in **Figure 2J–L** were generated using IL-1α at 3 ng/mL[9] in non-redox media at 1 mL per 10 cm^2 cell culture area.

4.2.3. Replace vmAstros in 37 °C/5% CO_2 incubator for 24 h. After 24 h, collect culture media into sterile microfuge tubes.

4.2.4. If ELISA will not be carried out immediately, snap freeze media samples by submerging microfuge tubes in liquid nitrogen and store at -80 °C for future analysis.

NOTE: The following protocol is optimized specifically for use with the IL-6 ELISA kit detailed in the **Table of Materials**. Antibodies and standards delivered as lyophilized powder in a new ELISA kit must be reconstituted prior to first use, and aliquoted for future use. The data sheet provided with the kit details the reagents and volumes required for reconstitution. The capture antibody must be reconstituted in PBS (without carrier protein).

4.2.5. Perform the ELISA in a 96-well plate format and calculate the volumes of reagents according to the wells used. On the day of ELISA, prepare capture antibody by diluting stock 1:120 in PBS. Coat the plate by loading 50 µL of capture antibody per well. Cover the plate with an adhesive strip and incubate at room temperature overnight.

4.2.6. Next day, wash plate. (3x with D-PBS-Tween (D-PBS with 0.05% Tween-20), 100 µL per well. Blot dry).

4.2.7. Block the plate by loading 150 µL D-PBS/1% BSA per well. Incubate at room temperature for at least 1 h. Wash plate as described.

4.2.8. Thaw samples on ice (this can take 1–2 h). Dilute samples 1:5 by loading 10 µL sample and 40 µL D-PBS/1% BSA. Vortex every sample prior to loading.

NOTE: It is necessary to dilute samples when carrying out an IL-6 ELISA, dilutions should be optimized.

4.2.9. Prepare the top standard (1,000 ρg/mL) by diluting stock 1:180 in D-PBS/1% BSA. Prepare 7 standards by carrying out a serial dilution of the top standard. Vortex between each dilution.

4.2.10. Add 50 µL of standards/samples to wells. Use D-PBS/1% BSA as the blank. Incubate at room temperature for 2 h on an orbital shaker to properly mix the diluted samples. Wash plate as described.

4.2.11. Prepare detection antibody by diluting stock 1:60 in D-PBS/1% BSA. Load 50 µL of detection antibody per well. Cover the plate and incubate at RT for 2 h. Wash plate as described.

4.2.12. Prepare streptavidin conjugated to horseradish peroxidase (Strep-HRP) by diluting stock 1:40 in D-PBS/1% BSA. Load 50 µL Strep-HRP per well and incubate at room temperature for 20 min in the dark. Wash plate as described.

4.2.13. Initiate the color reaction by loading 50 µL TMB substrate solution per well. Incubate at room temperature in the dark for 20 min (or until the standards and samples have developed a blue colour).

NOTE: TMB is stored at 4 °C but should be used at room temperature.

4.2.14. Stop the reaction by adding 25 µL stop solution (1 M H_2SO_4) per well and note the color change from blue to yellow.

4.2.15. Read the plate at 450 nm absorbance using a microplate reader. Set wavelength correction to 540 nm absorbance to maximize accuracy. Calculate the protein concentrations in the samples from the standard curve produced.

REPRESENTATIVE RESULTS:

Differentiation methodology and progression

Here we present the details of both the methods employed for the generation of vmAstros and the protocols used for their subsequent phenotypic characterization. The method for generation of vmAstros is made up of several distinct differentiation stages, which can be monitored by

microscopy and identifying distinct morphological characteristics (**Figure 1A–F**). A feeder-free hiPSC culture (**Figure 1B**) is exposed to specific factors to induce their differentiation toward a neural lineage (LDN193189, SB431542), specifically of the ventral midbrain (CHIR99021, SHH-C24ii). This results in the generation of a culture of vmNPCs, which are morphologically distinct from hiPSCs—the vmNPCs are less rounded than hiPSCs and vmNPCs have an elongated polygonal or triangular shape, typical of neural progenitors (**Figures 1B,C**). The morphological distinction is apparent from day 7–10 onward. When vmNPCs are passaged, similar to hiPSCs, we aim to maintain them as small cell clusters rather than single cells to increase cell survival (**Figure 1D**). However, whereas hiPSCs when passaged quickly form and remain as distinct colonies, vmNPCs readily form a monolayer (**Figure 1C**). From day 20 onward, vmNPCs can be used to generate midbrain dopaminergic neurons (**Figure 1A**), which we have previously published[30,31].

Our strategy to generate vmAstros from the vmNPCs relied on an understanding of the developing embryonic brain, the acquisition of astroglial fate in the embryo, and also how this has been applied to ex vivo neural progenitors and hiPSCs to generate astrocytes[22,23,25,32–37]. Elongated time in culture together with the activation of specific signaling pathways has been demonstrated to be required by mammalian NPCs to recapitulate the timing enabling the shift in neuronal potency toward the astroglial lineage in vitro[6,21–23,32]. Therefore, we used LIF and EGF to support the elongated expansion of the cultures from day 30–90 (**Figure 1A**). Both JAK/STAT signaling downstream of LIF, and EGF signaling are inducers of astroglial identity and also selectively act as mitogens on astroglial progenitors[22,23,25,33,34]. Media components for the culture of vmAPCs are modified from those demonstrated by[22] to support the generation of astrocytes from hiPSCs.

During the EGF/LIF mediated expansion, period cells are referred to as vmAPCs (**Figures 1A,E**). We expect that between days 50 and 90 to culture the vmAPCs in 175 cm^2 tissue culture flasks, passaging at ratios between 1:4 and 1:6 every 4 days, thus enabling rapid expansion of vmAPCs, which can be cryopreserved for future use.

From day 90 onward, vmAstros are generated from vmAPCs via the application of BMP4 in combination with LIF (**Figure 1F**). BMP signaling is required in vivo for mature astrocyte differentiation and recapitulates this effect in vitro[21,23,37,38]. In the culture flask, mature vmAstros appear larger than vmAPCs (**Figure 1F**).

The protocol detailed here has been carried out over six independent repeats, reproducibly generating vmAstros from the hiPSC line NAS2 and AST23. In addition, the generation of vmNPCs (for producing ventral midbrain dopamine neurons) has been carried out on multiple hiPSC and hESC lines as detailed in[30].

Characterization of vmAstros differentiation and phenotype

The ventral midbrain identity of the vmNPCs was confirmed by co-expression of the neural progenitor marker Musashi1 (MSI1) and the ventral midbrain transcription factor FOXA2 (**Figure 2A**). vmNPCs readily generate midbrain dopamine neurons, which co-express FOXA2 and dopaminergic marker tyrosine hydroxylase (TH) (**Figure 2B**). Expansion of vmNPCs in the

presence of EGF and LIF leads to the appearance of vmAPCs (**Figure 2C**). From day 90 onward of the protocol, vmAPCs are exposed to BMP4 in combination with LIF to induce maturation into vmAstros (**Figure 2D**). Immunocytochemistry confirmed co-expression of the ventral midbrain transcription factors LMX1A, LMX1B, and FOXA2 with the astrocyte marker S100β (**Figure 2E–G**). vmAstros also express the mature astrocyte marker GFAP (**Figure 2H**) and the novel marker CD49f, which has been shown to be specific to mature, functional astrocytes[39,40] (**Figure 2I**). Together these results confirm that treatment with BMP4 and LIF induces a mature astrocyte identity, as demonstrated both in vivo and in vitro, and that mature vmAstros maintain the regional ventral midbrain identity acquired in the primary stages of the differentiation protocol[21,37,38] (**Figure 2E–I**).

To confirm that the vmAstros are capable of neuroinflammatory modulation in line with their in vivo counterparts, we characterized their response to cytokine exposure. Exposure of vmAstros to the cytokine IL-1α for 24 h resulted in morphological changes similar to those demonstrated by ex vivo reactive mouse astrocytes[9] (**Figure 2J,K**). Specifically, upon addition of IL-1α, a large proportion of the vmAstros demonstrated a smaller, rounded cell body with multiple projections (**Figure 2K**). To confirm that these changes were representative of a reactive astrocyte phenotype in response to the neuroinflammatory stimuli, we measured the level of IL-6 secreted by the vmAstros. Increased IL-6 secretion is an indicator of reactivity in astrocytes. We measured IL-6 levels by ELISA after a 24 h treatment with IL-1α, which confirmed a large and significant increase in secreted IL-6, thus confirming the vmAstros were demonstrating a reactive phenotype (**Figure 2L**).

FIGURE AND TABLE LEGENDS:

Figure 1: HiPSC differentiation into vmAstros. (**A**) A schematic representation of the optimized protocol to generate vmAstros from hiPSC. The protocol is made up of distinct stages; first a neural, ventral midbrain fate is through dual-SMAD inhibition (with SB431542 and LDN193189) in combination with ventral midbrain patterning molecules (SHH (C24ii) and CHIR99021). vmNPCs proliferate rapidly in the absence of any exogenous mitogens during the vmNPC expansion stage. The addition of BDNF, GDNF, and ascorbic acid promotes survival of vmNPCs, supporting an increase in cell number. Addition of EGF and LIF sustains proliferation and promotes acquisition of astroglial fate over an extended culture period. After a minimum of 90 days from the initial hiPSC, vmAPCs form mature vmAstros upon exposure to BMP4. (**B–F**) Images of cells as they should appear at different stages in the protocol. hiPSCs and vmNPCs cells are passaged as small clusters, rather than single cells (**C**). Scale bars: B = 500 μm; C = 250 μm; D & E = 200 μm.

Figure 2: Characterization of the phenotype of intermediate cells and vmAstros generated using the described protocol, confirming a ventral midbrain identity. (**A**) Immunocytochemistry demonstrated vmNPCs express the neural progenitor marker MSI1 (red) and the ventral midbrain transcription factor FOXA2 (green). (**B**) vmNPCs are capable of generating midbrain dopamine neurons co-expressing dopaminergic marker tyrosine hydroxylase (TH; red) and ventral midbrain transcription factor FOXA2 (green) (previously published in[30]). (**C**) High magnification phase contrast images show the morphology of vmAPCs compared to (**D**) the mature vmAstros, which

656 have a larger area from nucleus to cell membrane. Immunocytochemistry demonstrated mature
657 vmAstros co-express astrocyte marker S100β and ventral midbrain markers LMX1A (**E**), LMX1B
658 (**F**), and FOXA2 (**G**). (**H,I**) vmAstros express GFAP and CD49f, which are associated with a mature
659 astrocyte phenotype. (**J,L**) Representative images demonstrating the morphology of untreated
660 cultures of vmAstros (**J**), compared to those exposed to IL-1α for 24 h (**K**). Exposure to IL-
661 1α resulted in clear morphological changes **(K)**. **(L)** In response to IL-1α, vmAstros significantly
662 increased secretion of IL-6, indicating vmAstros are generating a reactive phenotype in response
663 to neuroinflammatory stimuli (n = 3 independent experiments, SEM, unpaired *t*-test p = 0.0227).
664 All immunofluorescence images were taken on a confocal microscope. Scale bars: A, B, I = 50 μm;
665 C, D = 100 μm; E, F = 25 μm; G,H = 100 μm.
666
667 **DISCUSSION:**
668 This method for the generation of vmAstros from hiPSCs is highly efficient, generating pure
669 cultures of vmAstros, and being reproducible for the generation of vmAstros from different hiPSC
670 lines. This protocol was developed around the recapitulation of the developmental events
671 required in the embryo to correctly pattern the developing midbrain and generate
672 astrocytes and comprises three defined stages: 1) neural ventral midbrain induction to generate
673 vmNPCs, 2) generation and expansion of vmAPCs, and finally 3) maturation of vmAstros.
674
675 In our previous published work, we highlighted the importance of optimizing the concentrations
676 of CHIR99021 and SHH(C24ii) for each hiPSC line used to generate vmNPCs, to ensure optimal
677 expression of ventral midbrain markers[30,31]; 200 ng/mL SHH (C24ii) and 0.8 μM CHIR99021 yields
678 consistently reproducible results over multiple hiPSC lines. However, 300 ng/mL SHH (C24ii) and
679 0.6 μM CHIR99021 can be more efficacious for particular hiPSC lines but can also affect cell
680 survival[30,31]. Therefore, optimization by the user is recommended.
681
682 In developing this protocol for the generation of vmAPCs, it was apparent that cell density is
683 critical at all stages. In the vmNPC induction stage, cell density must remain high to support cell
684 survival, as vmNPC density below 75% leads to the death of vmNPCs in large numbers. The rate
685 of proliferation of the vmNPCs is dependent on the rate of proliferation of the parent hiPSC
686 culture and does vary between lines; however, to maintain a proliferative population, vmNPC
687 density must remain high. Therefore, we recommend that vmNPCs are passaged at conservative
688 ratios until the user has optimized the passaging regime. In contrast vmAPCs should be passaged
689 at high ratios, achieving a cell density of around 30-40% after passaging, and passaging as soon
690 as the cells are confluent. We found in our preliminary experiments that maintaining vmAPCs at
691 very high confluency leads to greater heterogeneity in the resulting vmAstros as indicated by
692 varied morphology and expression of astrocyte marker GFAP (data not shown). Micrographs of
693 appropriate cell densities are included in this protocol for reference.
694
695 Both the vmNPCs and vmAPCs are highly proliferative, generating large number of cells from a
696 relatively small starting population of iPSCs. For example, we usually begin this protocol with a
697 single 10 cm^2 dish of iPSCs and when we reach day 60, we would expect to culture the APCs in
698 175 cm^2 flasks, with each passage generating 4-6 new flasks and this rate of expansion would

continue until day 90. Extrapolating from this, at minimum we would have the ability to generate up to 4,000 flasks of vmAPCs. We cryopreserve the vmAPCs throughout this expansion period and thus can generate a large cryobank of cells for future generation of mature vmAstros. This is extremely advantageous as it enables high-throughput analysis required for applications such as drug screening.

The unique aspect of this protocol is the midbrain identity of the resulting vmAstros. In the brain, specific regional astroglial populations, similar to their neuronal counterparts, possess specific characteristics[25,26]. A major focus of Parkinson's research is the involvement of astroglial cells in neuroinflammation and how this influences disease progression. Neuroinflammation is present in early Parkinson's and many other injury or disease scenarios[12]. As part of the neuroinflammatory response, astrocytes transform in an attempt to protect neurons from damage—this is referred to as the "reactive astrocyte". However, reactive astrocytes are themselves neurotoxic in chronic diseases such as Parkinson's[9,11]. In animal and in vitro models of Parkinson's, reactive astroglial mediated neuroinflammation is a catalyst for neurotoxic α-synuclein pathology and neurodegeneration[9,14,41–43]. Therefore, we created an in vitro neuroinflammatory environment by treating vmAstros with pro-inflammatory cytokines IL-1α or IL-1β. In response, the vmAstros demonstrated significant morphological changes and we saw a significant increase in secretion of IL-6, which is also elevated in Parkinson's and is widely used as a measure of astrocyte reactivity.

In conclusion, this protocol provides a reproducible and efficient method to generate large numbers of hiPSC-derived vmAstros, which demonstrate a phenotype that parallels their in vivo counterparts in the ventral midbrain. This protocol is therefore highly applicable to high-throughput applications such as drug screening, which require large numbers of human cells. Recent work has highlighted the role of neuroinflammation in Parkinson's diseases, and how pharmacological targeting of astroglia influences neuroinflammation, which in turn modulates disease pathology[11]. As demonstrated, the vmAstros generated with this protocol are appropriately responsive to neuroinflammatory stimulation, providing a comprehensive cellular model with which to study astroglial involvement in Parkinson's disease.

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The authors have nothing to disclose.

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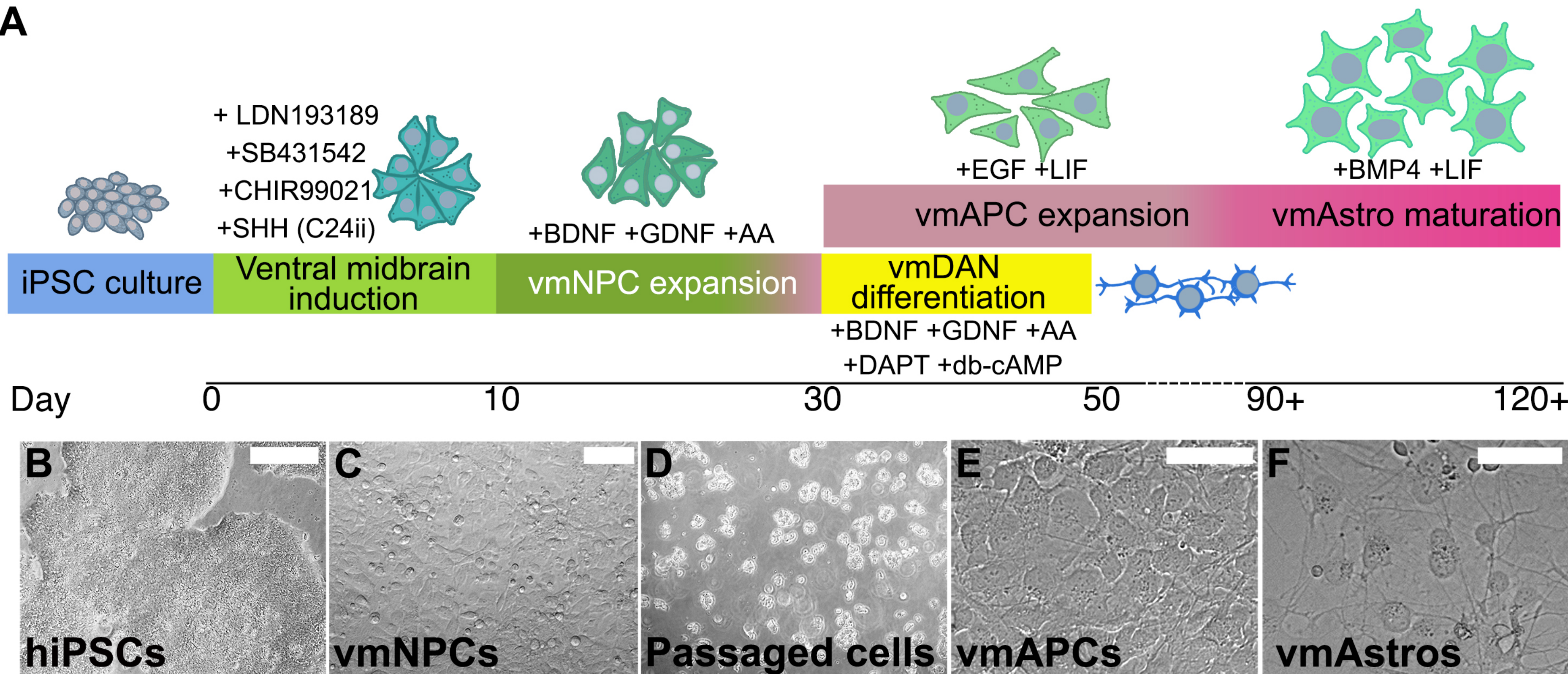
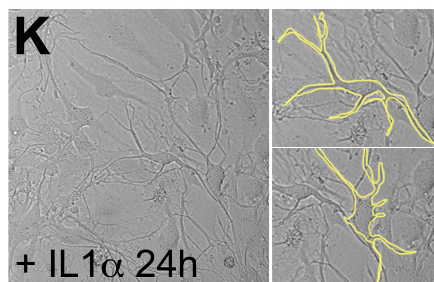
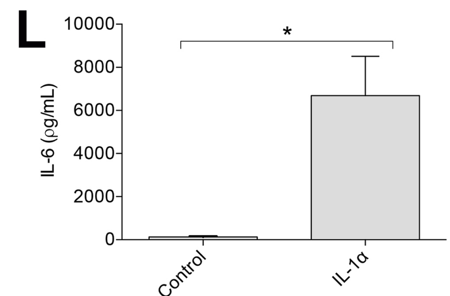
844


A
+ LDN193189
+SB431542
+CHIR99021
+SHH (C24ii)
+BDNF +GDNF +AA
+EGF +LIF
+BMP4 +LIF
iPSC culture
Ventral midbrain induction
vmNPC expansion
vmAPC expansion
vmAstro maturation
vmDAN differentiation
+BDNF +GDNF +AA
+DAPT +db-cAMP
Day
0
10
30
50
90+
120+
B
hiPSCs
C
vmNPCs
D
Passaged cells
E
vmAPCs
F
vmAstros

A FOXA2 MSI1
B FOXA2 TH
C vmAPC d90
D vmAstro
E S100β LMX1A DAPI OVERLAY
F S100β LMX1B DAPI OVERLAY
G S100β FOXA2 DAPI OVERLAY
H GFAP FOXA2 DAPI OVERLAY
I S100β CD49f DAPI OVERLAY





Reagents	**Company**	**Catalogue Number**	**Comments**	
0.2M Tris-Cl (pH 8.5)	n/a	n/a	Made up from Tris base and plus HCl	
0.5M EDTA, PH 8	ThermoFisher	15575-020	1:1000 in D-PBS to 0.5 mM final	
1,4-diazabicylo[2.2.2]octane (DABCO)	Sigma	D27802-	25 mg/ml in Mowiol mounting solution	
13 mm coverslips	VWR	631-0149		
2-Mercaptoethanol (50 mM)	ThermoFisher	31350010		
Accutase	ThermoFisher	13151014		
Advanced DMEM/F12	ThermoFisher	12634010	Has 1x NEAA but we add to final concentration of 2x (0.2 mM)	
Ascorbic acid	Sigma	A5960	200 mM stock, 1:1000 to 200 μM final	
B27 Supplement	ThermoFisher	17504-044	50x stock	
BSA	Sigma	5470		
Cell freezing media	Sigma	C2874	Cryostor CS10	
Cell freezing vessel	Nalgene	5100-0001		
CHIR99021	Axon Medchem	1386	0.8 mM stock, 1:1000 dilution to 0.8 μM final	
Cryovials	Sigma	CLS430487		
DAPI	Sigma	D9542	1 mg/ml, 1:10,000 to 100ng/ml final (in PBS)	
DMEM/F12 + Glutamax	ThermoFisher	10565018		
Dulbeccos-PBS (D-PBS without Mg or Ca)	ThermoFisher	14190144	pH 7.2	
E8 Flex medium kit	ThermoFisher	A2858501		
Formaldehyde (36% solution)	Sigma	47608		
Geltrex	ThermoFisher	A1413302	1:100 or 1:400 in ice-cold DMEM/F12	
Glutamax	ThermoFisher	35050038	2 mM stock (1:200 in N2B27, 1:100 in ASTRO media to 20 μM final)	
Glycerol	Sigma	G5516		
Human BDNF	Peprotech	450-02	20 μg/ml stock, 1:1000 to 20 ng/ml final	
Human BMP4	Peprotech	120-05	20 μg/ml stock, 1:1000 to 20 ng/ml final	
Human EGF	Peprotech	AF-100-15	20 μg/ml stock, 1:1000 to 20 ng/ml final	
Human GDNF	Peprotech	450-10	20 μg/ml stock, 1:1000 to 20 ng/ml final	
Human insulin solution	Sigma	I9278	10 mg/ml stock, 1:2000 to 5 μg/ml final	
Human LIF	Peprotech	300-05	20 μg/ml stock, 1:1000 to 20 ng/ml final	
IL-6 ELISA kit	Biotechne	DY206		
Isopropanol	Sigma	I9516-4L	For filling Mr Frosty cryostorage vessel	
LDN193189	Sigma	SML0559	100 μM stock, 1:10,000 dilution to 10 nM final	
Mowiol 40-88	Sigma	324590		
N2 Supplement	ThermoFisher	17502048	100x stock	
NEAA	ThermoFisher	11140035	10 mM stock, 1:100 to 0.1 mM final	
Neurobasal media	ThermoFisher	21103049		
Normal Goat serum	Vector Labs	S-1000-20		
Revitacell	ThermoFisher	A2644501	100x stock, 1:100 to 1x final	
SB431542	Tocris	1614	10 mM stock, 1:1000 dilution to 10 μM final	
SHH-C24ii	Biotechne	1845-SH-025	200 μg/ml stock, 1:1000 to 200 ng/ml final	
Tris-HCl	Sigma	PHG0002		
Triton-X	Sigma	X100		
Tween-20	Sigma	P7949		
Vitronectin	ThermoFisher	A14700	1:50 in D-PBS	

Table 1. Reagent preparation for hiPSC differentiation into ventral midbrain astrocytes (vmAstros)

Cell culture media

		% of final
N2B27 media		
	Neurobasal media supplemented with 1% glutamax	48.65
	DMEM/F12 + glutamax	48.65
	Non-essential amino acids	1
	β–mercaptoethanol	0.15
	B27	1
	N2	0.5
	Human Insulin solution (10 mg/mL)	0.05
		100
ASTRO media (based on[22])		
	Advanced DMEM/F12	96.6
	Non-essential amino acids (in addition to those in Advanced DMEM/F12)	1
	Glutamax	1
	N2	1
	B27	0.4

Addtional culture media additives

Reagent	Supplied as	Stock concentration	Reconstitute in	Details	Storage	Final concentraion in media
Ascorbic acid	powder	200 mM	filter sterilized water	0.0284 mL per 1 mg	Aliquot and store at -20 °C	200 μM
BDNF	lyophilized powder	20 μg/mL	filter sterilized D-PBS with 0.1% BSA	10 ml per mg	Aliquot and store at -20 °C	20 ng/mL
BMP4	lyophilized powder	20 μg/mL	filter sterilized D-PBS with 0.1% BSA	10 ml per mg	Aliquot and store at -20 °C	20 ng/mL
EGF	lyophilized powder	20 μg/mL	filter sterilized D-PBS with 0.1% BSA	10 ml per mg	Aliquot and store at -20 °C	20 ng/mL
GDNF	lyophilized powder	20 μg/mL	filter sterilized D-PBS with 0.1% BSA	10 ml per mg	Aliquot and store at -20 °C	20 ng/mL
LDN193189	lyophilized powder	1 mM	DMSO	2.085 mL per mg	Aliquot and store at -20 °C	100 nM
LIF	lyophilized powder	20 μg/mL	filter sterilized D-PBS with 0.1% BSA	10 ml per mg	Aliquot and store at -20 °C	20 ng/mL
SB431542	lyophilized powder	10 mM	DMSO	0.239 mL per mg	Aliquot and store at -20 °C	10 μM
SHH-C24ii	lyophilized powder	200 μg/mL	filter sterilized D-PBS with 0.1% BSA	1 ml per mg	Aliquot and store at -20 °C	20 ng/mL

Antibodies for immunocytochemistry	Company	Catalogue Number	Host species	Dilution Factor
Antibody against S100b	Sigma	SAB4200671	Mouse	1:200
Antibody against FOXA2	SCBT	NB600501	Mouse	1:50
Antibody against LMX1A	ProSci	7087	Rabbit	1:300
Antibody against LMX1A	Millipore	AB10533	Rabbit	1:2000
Antibody against LMX1B	Proteintech	18278-1-AP	Rabbit	1:300
Antibody against GLAST	Proteintech	20785-1-AP	Rabbit	1:300
Antibody against GFAP	Dako	Z0334	Rabbit	1:400
Antibody against CD49f	Proteintech	27189-1-AP	Rabbit	1:100
Antibody against MSI1	Abcam	ab52865	Rabbit	1:400
Alexa Fluor 488 Goat Anti-Rabbit	ThermoFisher	A32731	Goat	1:500
Alexa Fluor 488 Goat Anti-Mouse	ThermoFisher	A32723	Goat	1:500
Alexa Fluor 568 Goat Anti-Rabbit	ThermoFisher	A11036	Goat	1:500
Alexa Fluor 488 Goat Anti-Mouse	ThermoFisher	A11031	Goat	1:500