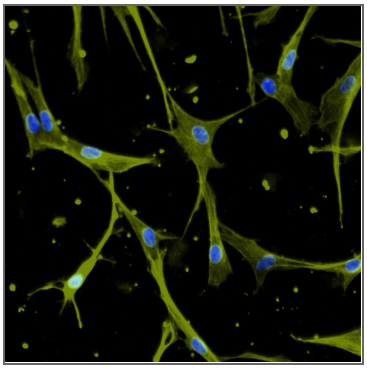

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Differentiation of Astrocytes from Human iPSC-derived NPCs

Balazs Szeky[1], Anita Feher[1], Melinda Zana[1], Radek Kucera[2,3], Jan Lochman[4,5], Andras Dinnyes[1,6]

[1]Biotalentum Ltd, 2100, Godollo, Hungary;
[2]Department of Pharmacology and Toxicology, Faculty of Medicine in Pilsen, Charles University, Alej Svobody 1655/76, 32300 Pilsen, Czech Republic;
[3]Department of Immunochemistry Diagnostics, University Hospital Pilsen, 30955 Pilsen, Czech Republic 5 Laboratory of Neurobiology and Pathological Physiology, Institute of Animal Physiology and Genetics, Czech Academy of Sciences, Brno, Czech Republic;
[4]Department of Biochemistry, Faculty of Science, Masaryk University, Kotlářská 2, 61137 Brno, Czech Republic;
[5]Laboratory of Neurobiology and Pathological Physiology, Institute of Animal Physiology and Genetics, Czech Academy of Sciences, Brno, Czech Republic;
[6]Department of Physiology and Animal Health, Institute of Physiology and Animal Nutrition, Hungarian University of Agriculture and Life Sciences, 2100, Godollo, Hungary

Biotalentum Ltd

Balazs Szeky
Biotalentum Ltd

ABSTRACT

Astrocytes are multifunctional glial cells of the central nervous system (CNS). They play essential roles in the metabolic support of neurons, synaptic transmission, maintaining blood-brain-barrier and immunological homeostasis. On the other hand, distinct subtypes of astrocytes play central roles in drug metabolism, neuroinflammation and in the exacerbation of neurodegenerative disorders. Understanding astrocye-mediated mechanisms of different CNS pathologies requires a reliable in vitro model system recapitulating in vivo astrocyte phenotypes and functionalities. To provide such models, we developed an efficient, time- and cost-effective method for the generation of functional astrocytes with high purity from human induced pluripotent stem cell (iPSC)-derived neural progenitor cells (NPCs).

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MATERIALS

See the appropriate paragraphs of the method for product informations.

SAFETY WARNINGS

Biosafety level 1. Human cells should be cultured under aseptic conditions in a BSL2 laminar flow.

ETHICS STATEMENT

Our research fully adhered to international ethical standards and guidelines for the establishemnt and maintenance of human iPSC liness, and iPSC-derived cell lines.

Preparation of Coated Plates and Solutions

1 Preparation of Coated Plates:

Neural progenitor cells can be grown on poly-L-ornithine/Laminin (POL) coated plates, whereas astroglial cells and astroglial progenitors must be seeded into Matrigel coated plates. Reagents required for preparing POL and matrigel plate coatings are described below.

1.1 Coating of Plates With POL

2h

	A	B	C	D
	Reagent	Manufacturer	Cat#	Dilution
	Poly-L-ornithine	Merck	P4957	1:5 (in PBS)
	Laminin	Sigma Aldrich	L2020	1 ug/cm2

I. Prepare the desired plate format for POL coating

II. Prepare 5-times diluted poly-L-ornithine solution in PBS. Pipette the proper volume into the desired number of wells, incubate at 37 °C for 01:00:00 .

III. Prepare 1 µg /cm^2 laminin solution On ice .

IV. Aspirate the Poly-L-ornithine from the plates. Wash the poly-L-ornithine-coated wells with PBS, then pipette the laminin solutions into the wells using chilled serological tips.

V. Seal the coated plates with parafilm, and store them at 4 °C overnight. Incubate the POL-coated plates at 37 °C for at least 01:00:00 before use.

1.2 Coating of Plates With Matrigel

30m

hESC qualified Matrigel (Corning, #CLS354277) must be kept on ice to prevent condensation into gel phase. Matrigel stock solutions can be used only when the solution becomes fully liquid. Matrigel must be diluted in ice-cold DMEM/F12 (Gibco, #11330032) medium.

I. Prepare the desired plate format for Matrigel coating

	A	B
	Plate format	Coating volume, mL/well
	6-well plate	1.5 mL
	24-well plate	0.5 mL
	96-well plate	0.1 mL

II. Prepare Matrigel solution and DMEM/F12 On ice . Add 25 ul of matrigel into every 1.5 mL of DMEM/F12.
III. Pipette the prepared Matrigel working solution into the wells of the plate(s) using chilled serological tips. Incubate the plates at 37 °C for at least 00:30:00 before use. For extended storage, seal the plates with parafilm and store at 4 °C . Plates can be stored up to 14 days.

2 Preparation of Cell Culture Mediums

Neural maintenance medium (NMM) must be prepared for NPCs. NMM is completed with [M] 10 ng/mL epidermal growth factor (EGF) and [M] 10 ng/mL basic fibroblast growth factor (bFGF).
Astrocyte induction medium (AIM) is used for astroglial progenitor cell (APC) enrichment from NPCs.
Astrocyte maturation medium (AMM) is used for astrocyte maturation from APCs. AMM is completed with [M] 20 ng/mL ciliary neurotrophic factor (CNTF).

2.1 Preparing NMM

Mix the components of NMM (listed in the table below) in a bottletop filter using filtered pipette tips and serological pipettes. Apply vacuum tube to the filter, turn on vacuum pump and sterile

filter the solution. store NMM at 4 °C .

	A	B	C	D
	Component	Manufacturer	Stock concentration	Final Amount/Dilution
	DMEM/F12 with GlutaMax	Gibco		50% (%vol)
	Neurobasal Medium	Gibco		50% (%vol)
	B27	Gibco	50X	1X
	N2	Gibco	100X	1X
	GlutaMax	Gibco	100X	0.5X
	Non-Essential Amino Acid Solution	Merck	100X	1X
	Pen/Strep	Gibco	100X	1X
	EGF	Thermo Fisher	0.1 mg/mL	10 ng/mL
	bFGF	Thermo Fisher	0.1 mg/mL	10 ng/mL

2.2 Preparing AIM

Mix the components of AIM (listed in the table below) in a bottletop filter using filtered pipette tips and serological pipettes. Apply vacuum tube to the filter, turn on vacuum pump and filter the solution. Store AIM at 4 °C .

	A	B	C	D
	Component	Manufacturer	Stock Solution	Final Amount/ Dilution
	Astrocyte growth Medium	ScienCell		
	Foetal Bovine Serum (FBS)	ScienCell		2% (vol%)
	Pen/Strep	Gibco	100X	1X

2.3 Preparing AMM

Mix the components of AMM (listed in the table below) in a bottletop filter using filtered pipette

tips and serological pipettes. Apply vacuum tube to the filter, turn on vacuum pump and filter the solution. Store AMM at 4 °C .

	A	B	C	D
	Component	Manufacturer	Stock Solution	Final Amount/Dilution
	Astrocyte Growth Medium	ScienCell		
	CNTF	Peprotech	0.01 mg/mL	20 ng/mL
	Pen/Strep	Gibco	100X	1X

Neural Progenitor Cell Expansion

2w

3 Thawing NPCs

3.1 Prepare POL-coated plates and NMM before thawing NPCs. Prepare 5 mL NMM into a 15 mL Falcon tube.

3.2 Thaw one vial of frozen NPCs under sterile cell culture hood in sterile gloves. Pipette the cell suspension gently into the falcon tube containing the 5 mL NMM.

3.3 Centrifuge the cell suspension: 1000 rpm, 00:03:00 . Discard the supernatant. 3m

3.4 Resuspend the pellet in NMM, complete with EGF and bFGF. Pipette the suspension into the wells of the POL-coated plate

3.5 Put the plate into a cell culture incubator, (37 °C , 5% CO_2). Change medium next day to complete NMM.

3.6 Change medium every other day onwards. Passage NPCs upon reaching confluence.

4 Passaging NPCs

4.1 Prepare coated POL plate, complete NMM and Accutase at Room temperature for passaging of NPCs.

4.2 Wash the cells with PBS, (2 mL /well for 6-well plates). Avoid scratching the cell monolayer.

4.3 Add Accutase, (1 mL /well for 6-well plates). Incubate at 37 °C for 00:03:00 . Afte 3m the incubation, the rollup of the cells can be observed under the microscope.

4.4 Add NMM (2 mL /well for 6-well plates) to stop the accutase digestion. Dissociate the monolayer by pipetting. Transfer the cell suspension into the Falcon tube.

4.5 Centrifuge the cell suspension: 1000 rpm, 00:03:00 . Discard the supernatant, except 200 µL . Resuspend the cells. 3m

4.6 Add completed NMM . Perform cell counting. Dilute the cell suspension with complete NMM to

obtain 75.000-100.000 cells/cm^2 (depending on further experiments) for each well of the POL-coated plate, (1 million cells/well for 6-well plates).

4.7 Pipette the cell suspension into the POL-coated plates, (2 mL /well for 6-well plates). Distribute the cells by gentle shaking. Put the plates into a sterile cell culture incubator, (37 °C , 5% CO_2).

4.8 Change the medium next day to complete NMM. Change medium every other day onwards until the next passage.

5 Plating for Astrocyte Induction

5.1 By the second passaging of the NPCs, prepare Matrigel-coated plates, AIM and Accutase.

5.2 Passage and plate cells as described in steps 4.1-4.6.

5.3 Perform cell counting. Dilute the cell suspension with complete NMM to obtain 25.000-75.000 cells/cm^2 for each well of the Matrigel-coated plate.

5.4 Pipette the suspension into the matrigel coated plate (2mL/well for 6-well plates). Distribute the cells by gentle shaking. Put the plate into a sterile cell culture incubator, (37 °C , 5% CO_2).

Astroglial Progenitor Cell Induction

6 **Starting astrocyte induction at Day 0.** Next day after the second NPC passaging, replace completed

NMM intro AIM, (2mL/well for 6-well plates). Change the medium every other day **at least for 21 days.** Passage the APCs upon reaching confluence.

7 Passaging APCs

7.1 Prepare Matrigel-coated plate, AIM and Accutase at Room temperature for passaging of APCs.

7.2 Wash the cells with PBS, (2 mL /well for 6-well plates). Aspirate the PBS. Avoid scratching the cell monolayer.

7.3 Add Accutase, (1 mL /well for 6-well plates). Incubate at 37 °C for 00:03:00 - 00:07:00 until the cells begin to roll up.

7.4 Add AIM (2 mL /well for 6-well plates) to stop the accutase digestion. Dissociate the monolayer and add the 1mL cell suspension into a 15 mL Falcon tube.

7.5 Wash with 1 mL AIM and collect the rest of the cell suspension into the falcon tube.

7.6 Centrifuge the cell suspension: 1000 rpm, 00:03:00 . Discard the supernatant, except 200 µL . Resuspend the cells using 200 uL pipettes with filtered tips. 3m

7.7 Perform cell counting. Dilute the cell suspension with AIM to obtain 25.000-75.000 cells/cm^2 for

each well of the Matrigel-coated plate, (0.5 million cells/well for 6-well plates).

7.8 Pipette the cell suspension into the Matrigel-coated plates. Distribute the cells by gentle shaking. Put the plates into a sterile cell culture incubator, (37 °C , 5% CO_2).

7.9 Change the medium next day to AIM. Change medium every other day onwards until the next passage.

Astrocyte Maturation

8 **Initiating Astrocyte Maturation from APCs.**

8.1 On the 21st day of differentiation, prepare Matrigel-coated plates, AIM, AMM and Accutase for the passaging of APCs.

8.2 Passage APCs as described in steps 7.1-7.9. Change medium to AMM completed with [M] 20 ng/mL CNTF (2 mL/well for 6-well plates) next day, and every other day onwards **at least for 42 days**.