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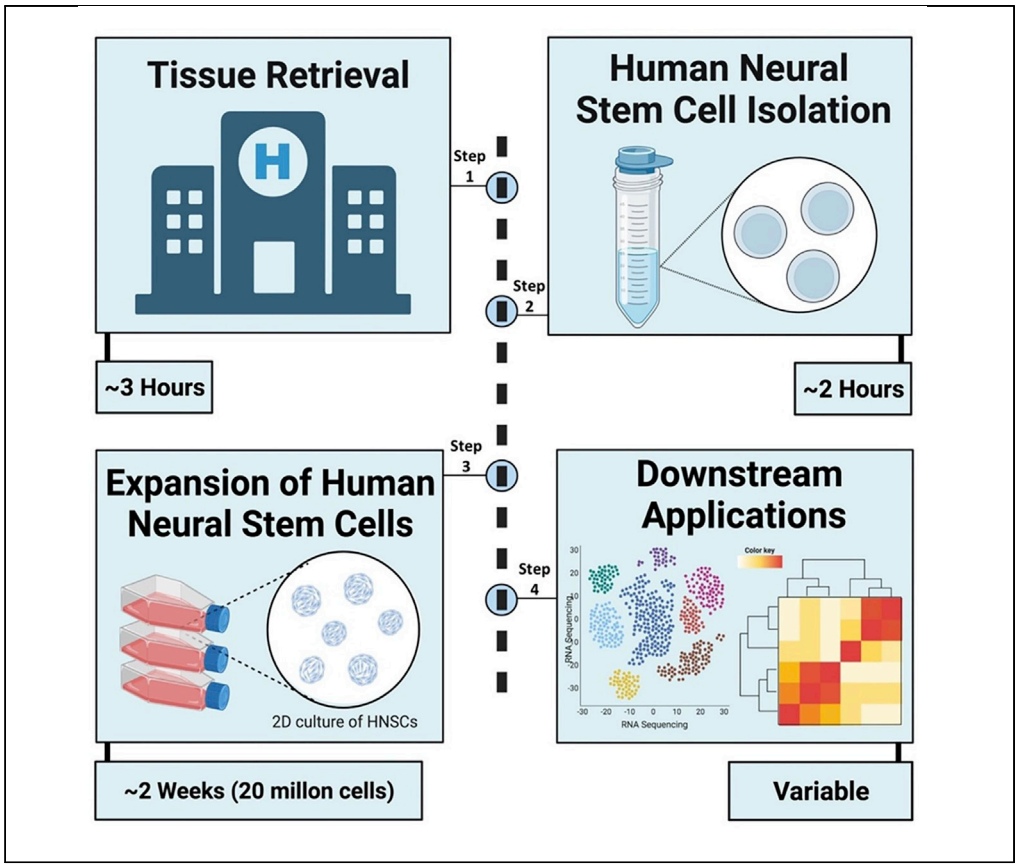
Derivation and culturing of neural stem cells from human embryonic brain tissue



Human neural stem cells (hNSCs) are a valuable tool in brain cancer research since they are used as a normal control for multiple assays, mainly pertaining to toxicity. Here, we present a protocol to safely and successfully derive and culture hNSCs *in vitro* from human embryonic brain tissue. We describe the steps to dissociate embryonic brain tissue and culture hNSCs, followed by the procedure to expand hNSCs. These cells can be used for downstream applications including RNA-seq and omics studies.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Derivation and cryopreservation of hNSCs from human embryonic tissue

Long-term culture and expansion of primary hNSC cells

Feasible to generate up to 200 million human neural stem cells in 1 to 2 months

Can be used for downstream applications such as RNAseq and omics techniques

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Derivation and culturing of neural stem cells from human embryonic brain tissue

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SUMMARY

Human neural stem cells (hNSCs) are a valuable tool in brain cancer research since they are used as a normal control for multiple assays, mainly pertaining to toxicity. Here, we present a protocol to safely and successfully derive and culture hNSCs *in vitro* from human embryonic brain tissue. We describe the steps to dissociate embryonic brain tissue and culture hNSCs, followed by the procedure to expand hNSCs. These cells can be used for downstream applications including RNA-seq and omics studies.

For complete details on the use and execution of this protocol, please refer to Venugopal et al. (2012b), Bakhshinyan et al. (2019), and Venugopal et al. (2012a).

BEFORE YOU BEGIN

Institutional permissions

Timing: Varies

Our protocol is approved by the Hamilton Health Sciences/McMaster Health Sciences Research Ethics Board and samples are collected from consenting patients as approved by the committees.

To set up collection and processing of embryonic brain tissues all relevant institutional permissions must be obtained in advance and tissues should be collected and processed following appropriate legislative and ethical approvals.

Embryonic brain tissue specimens should be collected from a gestational age of 8–11 weeks with no identified germline mutations. Embryonic brain specimens are identified by a nurse immediately after retrieval and should be immediately placed in a sterile container filled with phosphate-buffered saline and on ice during transport. Informed consent must be obtained to collect embryonic brain specimens. A pathologist must approve of and release the sample prior to culturing the tissue.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Healthy Human Embryonic Brain Tissue 8–11 gestational weeks	McMaster University	N/A
Chemicals, peptides, and recombinant proteins		
Liberase Blendzyme (2.5 mg/mL)	Roche	Cat. No. 05401119001
Red blood cell (RBC) lysis buffer Ammonium Chloride Solution	STEMCELL Technologies	Cat. No. 07850
PBS pH 7.4, with calcium and magnesium	Wisent Bioproducts	Cat. No. 311-011-CL
MycoZap prophylactic	Lonza	Cat. No. VZA-2031
Trypan blue	Thermo Fisher Scientific	Cat. No. 15250-061
TrypLE™ express enzyme (1X), phenol red	Thermo Fisher Scientific	Cat. No. 12605028
Poly-L-ornithine solution, 0.01%	Sigma-Aldrich	Cat. No. P4957
Laminin	BD Biosciences	Cat. No. 354232
Human Recombinant Epidermal growth factor	STEMCELL Technologies	Cat. No. 78006
Human Recombinant Basic Fibroblast Growth Factor	STEMCELL Technologies	Cat. No. 78003.2
100X Wisent Antibiotic/Antimycotic	Wisent	Cat. No. 450-115-EL
Heparin Solution	STEMCELL Technologies	Cat. No. 07980
NeuroCult NS-A proliferation kit (Human)	STEMCELL Technologies	Cat. No. 05751
Other		
50 mL sterile conical tubes	Thermo Fisher Scientific	Cat. No. 339652
CryoPure Tube 1.8 mL mix	Sarstedt	Cat. No. 72.379.992
Corning® CoolCell® Containers	Corning	Cat. No. 432000
Falcon® 70 µm Cell Strainer, White, Sterile, Individually Packaged, 50/Case	Corning	Cat. No. 352350
Countess™ 3 FL Automated Cell Counter + ABRC Extended Warranty	Thermo Fisher Scientific	Cat. No. A49893
Countess chamber slides	Invitrogen	Cat. No. C10228
Nunc™ EasYDish™ Dishes	Thermo Fisher Scientific	Cat. No. 150466
Shaking Incubator	Avantor VWR	Cat. No. 12620-90

MATERIALS AND EQUIPMENT

Neurocult complete (NCC) media		
Reagent	Final concentration	Amount
NeuroCult basal medium	N/A	443.3 mL
NeuroCult supplement	N/A	50 mL
Antibiotic/Antimycotic solution (100X)	1X	5 mL
MycoZap prophylactic (500X)	1X	1 mL
Heparin solution	0.0002% (w/v)	0.5 mL
EGF	20 ng/mL	0.1 mL
bFGF	10 ng/mL	0.1 mL
Total	N/A	500 mL

Storage: Store at 4˚C for up to 2 weeks. Store all reagents as per manufacturer's instructions.

STEP-BY-STEP METHOD AND DETAILS

Timing: 2 h

Dissociation of embryonic brain tissue and primary culture.

This section describes the derivation of viable hNSCs from embryonic brain tissue.

Note: This protocol should be performed under sterile conditions.

1. Add 200 μL Liberase Blendzyme to 15 mL of warmed PBS. It is recommended to use a 50 mL conical tube.

   Note: If immediate dissociation is not possible, sample can be dissected into smaller portions and frozen down in fetal bovine serum supplemented with 10% DMSO.

   Note: The amount of Liberase Blendzyme used will vary depending on the size of the tissue; 200 μL is suitable for a roughly 1cm by 1cm tissue sample. For smaller tissue samples, decrease the volume of **Liberase** Blendzyme used. For larger tissue samples, dissect the tissue into dime-sized portions and process separately until step 8. Liberase Blendzyme can be aliquoted and stored in -20°C and thawed prior to each use.

2. Wash embryonic brain tissue with phosphate buffered saline (PBS) to remove excess blood. Any pieces of hard tissue should be removed with sterile forceps and discarded.
3. Using scissors or a razor blade, disaggregate tissue into a slurry consistency ensuring sufficient solution volume to prevent tissues from drying out.
4. Transfer fragmented slurry into the pre-warmed PBS prepared in step 1.
5. Place the sample in a pre-warmed shaking incubator for 15 min.
6. Filter the sample into a new sterile 50 mL conical tube through a 70μm cell strainer. The neural stem cells (NSCs) will be transferred into the fresh tube.
7. Centrifuge the NSCs for 5 min at 290g at room temperature.
8. Remove the supernatant and resuspend the cell pellet in 4–12 mL of RBC lysis buffer.

   Note: The volume of RBC lysis buffer added will depend on the pellet size and number of red blood cells (i.e., if the pellet is white, 4 mL should suffice. If the pellet is a dark red colour, 12 mL should be used). **The** RBC lysis step can be repeated 1–2 more times if pellet is still pink or red in color (should be white).

9. Incubate the cells for 5 min at room temperature.
10. Add an equal volume of PBS and centrifuge the sample for 5 min at 290 *g*.
11. If the pellet is still red in color, repeat steps 8–10. Otherwise, proceed to step 12.
12. Remove the supernatant and wash the cells with 5 mL of sterile PBS. Centrifuge the sample for 5 min at 290 *g*.
13. Remove the supernatant and resuspend the cells in 10 mL NCC supplemented with Mycozap and transfer to 10 cm ultra-low plate (or 60mm plate if the pellet is smaller). Cells are expected to form neurospheres in 3–7 days (Figure 1).

    Note: As samples are collected from the hospital before being transferred for processing in sterile biosafety cabinets, there are risks of mycoplasma contamination during the handover. We recommend culturing the hNSCs in Mycozap media for 2 weeks prior to use for experiments. Mycozap can be aliquoted and stored at −20°C and thawed prior to each use.

14. If the color of the media begins to turn orange or yellow in color within the first week of culture, add fresh media to the existing media (i.e., do not change the media for the first week of culture).

    Note: It is recommended that media changes remain minimal in the initial stages when cell density is low as the hNSCs produce endogenous EGF and FGF that are beneficial in promoting cell proliferation and **viability**. Media should initially be supplemented only based on changes in acidity and as cell density increases with gradual increases in removing and replacing fresh media.

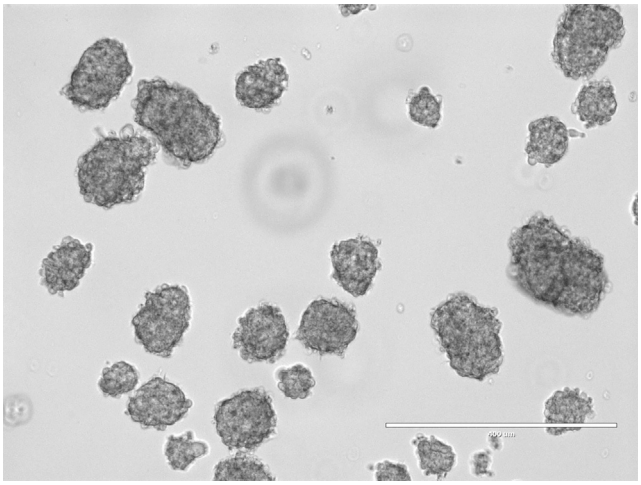

Figure 1. hNSC197 P11 neurospheres 10X grown in suspension in ultra-low binding plates

15. After 1–2 weeks in culture, NSCs can be cryopreserved and/or kept in culture and expanded on polyornithine-laminin PoL plates (see below for plate preparation details) for downstream applications.

Note: It is recommended to save multiple copies of early passage hNSCs (P1-P6) as long-term culture and multiple enzymatic passaging will eventually lead the hNSCs into senescence and/or differentiation. We do not recommend using hNSCs beyond 8–10 enzymatic passages, or after their growth is observed to be significantly slowed.

Cryopreservation of primary hNSC culture

Timing: <1 h

This section describes the cryopreservation of hNSCs.

Note: For cryopreservation of hNSCs in suspension follow steps 16–24, for cryopreservation of adherent hNSCs on PoL plates first follow steps 42–45 then return to step 18.

16. Pre-warm media in the 37°C water bath.
17. Collect hNSCs in suspension into 50 mL conical tube.
18. Rinse plate an additional 1–2 times with PBS collecting the additional rinses.
19. Centrifuge the sample for 5 min at 290 *g* at room temperature.
20. Resuspend cells in 1 mL of warm NCC media.
21. Assess cell viability using a dye exlusion test such as Trypan Blue solution.

Note: It is recommended to cryopreserve a minimum of 1×10^6 hNSCs per vial (ideally 2–5×10^6 per vial) to account for cell loss during the cell freezing and thawing process.

22. Transfer desired number of cells to cryovial(s) and supplement with 100 µL of DMSO ensuring that the final volume is 1 mL of 10% DMSO in NCC per vial.
23. Transfer vials to a freezing container and move the container into a −80°C freezer.
24. Transfer vials to liquid nitrogen after 24 h for long term storage.

Polyornithine-laminin (PoL) plate preparation

Timing: 24h

This section describes the preparation of PoL plates for efficient hNSC primary culture expansion.

Note: Prepare PoL plate 24 h prior to cell culture, it is highly recommended to grow hNSCs on PoL plates as the rate of expansion is the significantly faster when grown adherently as compared to hNSCs grown in suspension.

25. Dilute 0.01% poly-L-ornithine solution 1:5 in PBS without calcium and magnesium.
26. Coat entire surface of cell culture plate with diluted poly-L-ornithine solution and leave in the incubator for 1–3 h (suggested volumes are as follows: 7 mL for 100mm dish, 5 mL for T25, 2 mL for 60 mm dish).

Note: Laminin should be thawed on ice to avoid laminin solidifying, if laminin forms gel it is no longer viable.

27. Dilute laminin (stock is 1mg/mL) in PBS for final concentration of 5μg/mL.
28. Rinse plate gently with PBS then coat entire plate with diluted laminin solution (7 mL for 100 mm dish, 5 mL for T25, 2 mL for 60 mm dish).
29. Incubate plate overnight (16–24 h) at 37°C, 5% CO_2.

Note: Plate can be used right away or stored in 4°C with parafilm seal for up to 2 weeks.

In vitro hNSC primary culture propagation

Timing: 2 weeks for $\sim 2 \times 10^7$ cells from a freshly thawed early passage vial (less than P15) of hNSCs

This section describes the expansion of hNSCs for downstream applications.

30. Pre-warm media in 37°C water bath.
31. Retrieve hNSCs vial from liquid nitrogen and immediately transfer to a 37°C water bath.
32. As soon as vial is thawed, acclimate it with 1 mL of warm media in a drop wise manner and transfer to a 15 mL conical tube.
33. Centrifuge the cells for 5 min at 290 *g* at room temperature and discard the supernatant.
34. Resuspend cells in 1 mL of warm media, pipetting up and down gently 3–4 times.
35. Assess the cell viability with Trypan Blue on clean countess slides.
36. Plate hNSCs on PoL coated plates at approximately 5×10^4 cells per cm^2 (3×10^6 cells on a 100 mm dish) with appropriate media volume (approximately 8 mL total volume for a 100 mm dish).
37. Ensure even coating of cells across plate.
38. Incubate plate at 37°C, 5% CO_2 allowing cells to adhere for 24 h.
39. Replace half the media with warm fresh media the next day.
40. As cells propagate, increase the total volume of media in the culture plate ensuring daily media changes and avoiding full replacement of media at once unless passaging.
41. Cells can be passaged once they reach greater than 85% confluency (Figure 2).

Note: hNSCs should be ideally passaged at confluency (90–95%) to ensure higher seeding density and lower passage numbers.

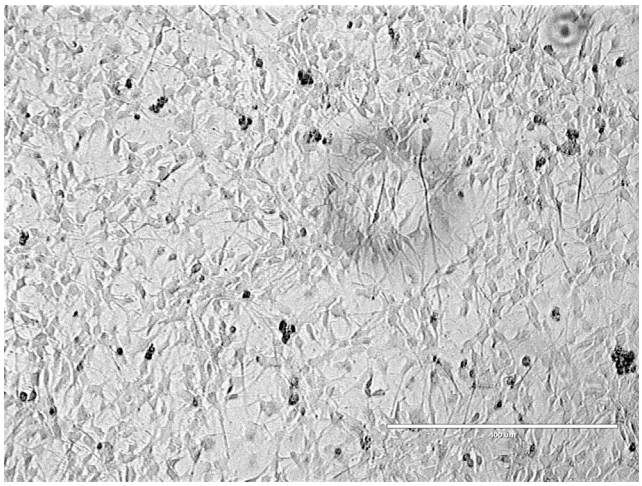

Figure 2. hNSC197 P11 10X adherently grown on PoL plate at 85% confluency

42. To passage cells, remove media and rinse plate gently with PBS rocking back and forth 2–3 times.
43. Remove PBS and add TrypLE until solution is covering entirety of the plate (approximately 1 mL for 100 mm dish, 0.5 mL for 6 well dish).
44. Incubate plate for 2–5 min at 37°C, 5% CO_2.
45. Once cell detachment is complete, add 1–2 mL of PBS to collect cells gently pipetting up and down to break up clumps and collect into a 15 mL conical tube.
46. Rinse plate an additional 1–2 times with PBS collecting the additional rinses.
47. Centrifuge the cells for 5 min at 290 *g* at room temperature.
48. Resuspend cells in 1 mL of warm media.
49. Assess the cell viability with Trypan Blue on clean countess slides.
50. Follow plating and upkeep instructions in steps 36–40.

Note: It is recommended hNSCs be plated onto tissue culture plates 24–48 h prior to functional/stem cell assays to form neural spheres as suspension culture.

EXPECTED OUTCOMES

The derivation of a viable primary neural stem cells should serve as a gold standard control for neuroscience and neurobiology research. Using neural stem cells as a gold standard control will streamline both target-based and drug discovery research initiatives by ensuring that novel therapies under investigation are not toxic to normal brain cells before moving to preclinical studies (Jakel et al., 2004). This will enable a quick and reliable method to determine the cyto- and neurotoxicity of proposed cellular therapies to treat CNS disease and drug target validation, among others. Alternatively, given the neural cells' plasticity and self-renewal capabilities, neural stem cells can be differentiated to generate other types of brain cells for research purposes. Generation of up to 200 million human neural stem cells are possible in a 1–2 months time frame with optimal culturing and passaging techniques and can be used for downstream techniques including RNAseq, metabolomics, proteomics, lipidomics, etc.

LIMITATIONS

Patients may choose to remove their sample from the study at any time, this can be mitigated by including multiple NSC lines in studies to limit risk of having to repeat an entire project. Neural stem cells are susceptible to environmental exposures and can enter senescence of terminal differentiation limiting the extent that the cells can proliferate. Phenotypic changes are irreversible, and cells should be replaced with a fresh vial. Notably, most neural stem cell lines begin to lose their

proliferation capacity at high passage numbers (~P20 onward) even with proper care and treatment. Researchers should be careful to ensure the neural stem cells used as normal control for experiments are kept in optimal conditions and truly represent a stem like population by performing functional stem cell assays and differentiation experiments prior to downstream experiments.

TROUBLESHOOTING

Problem 1

Cells are not expanding (step 40).

Potential solution

- Cells may be plated at a density that is too low; cells may be trypsinized and replated onto a smaller dish to increase cell density.
- Cells may be at a passage number that is too high; differentiated cells or cells undergoing senescence should be discarded and a fresh vial should be thawed for subsequent experiments.
- Cells may have been exposed to mycoplasma; media can be tested and, if positive for mycoplasma, Mycozap treatment can be initiated for a duration of 1–2 weeks until tests return negative, during this time contaminated incubators must be cleaned and cells must be quarantined until cells test negative.

Problem 2

Cell phenotype has changed from representative figures.

Potential solution

- Cells most likely have differentiated; thaw fresh vial replacing stock with passages.

Problem 3

Cells are not attaching uniformly (step 38).

Potential solution

- Incubator shelf may not be level; ensure the shelves are leveled.
- PoL plate may have dried up in certain areas due to insufficient solution volume; use a freshly made PoL plate.

Problem 4

There are a lot of floating dead cells/debris (step 40).

Potential solution

- Media may be acidic; replace with fresh warm media.
- Plates may have been left at room temperature for too long; return to incubator and keep handling to a minimum.
- Cells may have been exposed to mycoplasma; media can be tested and, if positive for mycoplasma, Mycozap treatment can be initiated for a duration of 1–2 weeks until tests return negative, during this time contaminated incubators must be cleaned and cells must be quarantined until cells test negative.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Sheila Singh (ssingh@mcmaster.ca).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze data, original hNSC derivation and differentiation assay findings were published in Venugopal et al., (2012b), Bakhshinyan et al. (2019), and Venugopal et al., (2012a).

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Y.S., A.K., and D.M. contributed to design and generation of manuscript; C.V. and S.K.S. provided feedback and guidance.

DECLARATION OF INTERESTS

S.K.S. is a scientific advisor for Century Therapeutics Inc., and her role in the company has been reviewed and is supported by McMaster University. C.V. is a member of the *STAR Protocols* advisory board.

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