Chapter 5

Culturing and Expansion of "Clinical Grade" Neural Stem Cells from the Fetal Human Central Nervous System

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Abstract

NSCs have been demonstrated to be very useful in grafts into the mammalian central nervous system to investigate the exploitation of NSC for the therapy of neurodegenerative disorders in animal models of neurodegenerative diseases. To push cell therapy in CNS on stage of clinical application, it is necessary to establish a continuous and standardized, clinical grade (i.e., produced following the good manufacturing practice guidelines) human neural stem cell lines.

In this chapter we will illustrate some of the protocols for the production and characterization routinely used into our GMP "cell factory" for the production of "clinical grade" human neural stem cell lines already in use in clinical trials on neurodegenerative diseases, particularly amyotrophic lateral sclerosis (ALS—Clinicaltrials.gov number NCT01640067) and secondary progressive multiple sclerosis (SPMS—Clinicaltrials.gov number NCT03282760).

Key words Clinical grade, Precursor cells, Human central nervous system, Neural stem cells, Therapy

1 Introduction

Somatic adult neural stem cells (NSCs) are undifferentiated cells that reside in specialized regions, namely, the niche, of the fetal and adult central nervous system (CNS); they possess lifelong self-renewal ability and a multipotent differentiation potential, given their ability to generate neurons, astrocytes, and oligodendrocytes. Reynolds and Weiss [1] have first demonstrated a stem cell niche in the mammals CNS. In particular, the finding of adult neurogenesis in the subventricular zone (SVZ), which leads to the generation of neural progenitors migrating to the olfactory bulbs and to the cortex, has favored the idea that newborn neurons might subserve cognitive functions and contribute to the homeostasis of the telencephalic-diencephalic area.

During the last decades, NSCs have been demonstrated to be very useful in grafts into the mammalian central nervous system to

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investigate the exploitation of NSC for the therapy of neurodegenerative disorders including both genetic diseases like metachromatic leukodystrophy (MLD), Huntington's disease (HD), and Alzheimer's disease (AD) (sporadic) and idiopathic diseases like Parkinson's disease (PD), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), and stroke [2–5].

The discovery of the existence of NSCs in the adult rodent brain followed by the development of a standardized and efficient protocol to isolate and safely expand NSCs also from humans by our group since 1999 [6, 7] has paved the way to the development of perspective cell therapy applications for neurodegenerative diseases.

In fact, pushed by the encouraging preclinical results into abovementioned disorders, cell therapy in CNS is reaching the stage of clinical application, with the first clinical trials already concluded in posttraumatic, postischemic, tumorigenic, or neurodegenerative disorders (*see* www.clinicaltrial.gov for all clinical trials underway or concluded) particularly in ALS, Parkinson, and multiple sclerosis diseases. A continuous and standardized, clinical grade (i.e., produced following the good manufacturing practice guidelines and approved by national and international regulatory agencies) of normal human NSCs would be of paramount importance in regenerative medicine field.

Our adult human hNSCs have now been serially expanded under chemically defined conditions and are being cryopreserved according to good manufacturing practice protocols (GMP) [8] by the cell factory: Laboratorio Cellule Staminali, Cell Factory and Biobanca, at the Azienda Ospedaliera Santa Maria, Terni, that has received formal approval and certification by the Agenzia Italiana del Farmaco (AIFA), protocol number aM 101/2010, also as updated in 2018 after AIFA inspection, now protocol number aM 54/2018, and in Unità Produttiva per Terapie Avanzate (UPTA) at the IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, that has received formal approval and certification by the Agenzia Italiana del Farmaco (AIFA), protocol number aM 118/2019.

Cells produced in the previous cited cell factory have already been used to perform experimental clinical trials on ALS and secondary progressive MS with encouraging safety results.

Indeed, none of the 18 recruited ALS patients for up to 60 months after surgery manifested severe adverse effects or increased disease progression because of the treatment. Eleven patients died, and two underwent tracheotomy as a result of the natural history of the disease. Moreover, we detected a transitory decrease in progression of ALS Functional Rating Scale Revised, starting within the first month after surgery and up to 4 months after transplantation. Our results show that transplantation of hNSC is a safe procedure that causes no major deleterious effects over the short or long term [9].

Also the 12 SPMS patients treated till now showed no severe adverse effects or increased disease progression, and the clinical and radiological evaluation of the disease are ongoing.

In order to certify these cells by the GMP standard, a panel of cellular, functional, and biochemical criteria must be met prior to cell release, which include, but are not limited to, karyotype analysis, stable differentiation and growth capacity, and lack of biological contamination by adventitious agents.

In our GMP facilities designed to produce human neural stem cells for advanced therapies, quality control is only part of overall quality assurance for cell lines which includes evaluation and quality control measures for cells and critical reagents coming into the laboratory; control of the laboratory environment, equipment, and procedures; control of data arising from cell culture; control of the delivery of research materials, including cells, to other laboratories; and traceability of raw material especially tissue from donors.

Four critical parameters are fundamental to assure the quality of any cell culture especially those performed in a GMP environment for human use:

1. Identity, i.e., the cells need to possess specific characteristics in order to be considered neural stem cells:
   (a) Self-Renewal: growth kinetic stable for an elevated number of passages in vitro.
   (b) Multipotency: The cells are able to differentiate into three neural lineages (astrocytes, neurons, and oligodendrocytes).
2. Microbiological safety, i.e., no microbiological contamination or endotoxins that could prime a dangerous overreaction from patient's immune system, must be detected in every and each moment of the cell culture. All the assays need to be performed according to official European Pharmacopoeia, current edition [10] in a GMP-approved microbiological laboratory:
   (a) Sterility (bacteria and fungi).
   (b) Mycoplasma.
   (c) Bacterial endotoxins.
   (d) Viral adventitious contamination.
   (e) BSE (at list risk analysis) contamination.
3. Purity and the maintenance of stable functional/genetic properties over passaging in vitro:
   (a) Growth curve: constant positive slope over passages.
   (b) Presence of neurons/astrocytes/oligodendrocytes upon differentiation assay.
   (c) Clonal efficiency: maintaining over passages the ability to form primary and secondary neurosphere when neural stem cells were seeded at low concentration.

(d) Karyology (healthy karyotype asset and deeper analysis like SKY or comparative genomic hybridization, detection of DNA polymorphisms, particularly a single nucleotide polymorphism (SNP), a variation at a single site in DNA that is the most frequent type of variation in the genome).

4. Tumorigenicity, i.e., cell lines not toxic or tumorigenic:
   (a) Growth factor dependence: the cells died into a few passages after EGF and bFGF removal from culture medium.
   (b) No tumor signs after transplantation into the brain of Nude mice (*see* Fig. 1). The cells are able to migrate, differentiate, and integrate into host tissue.

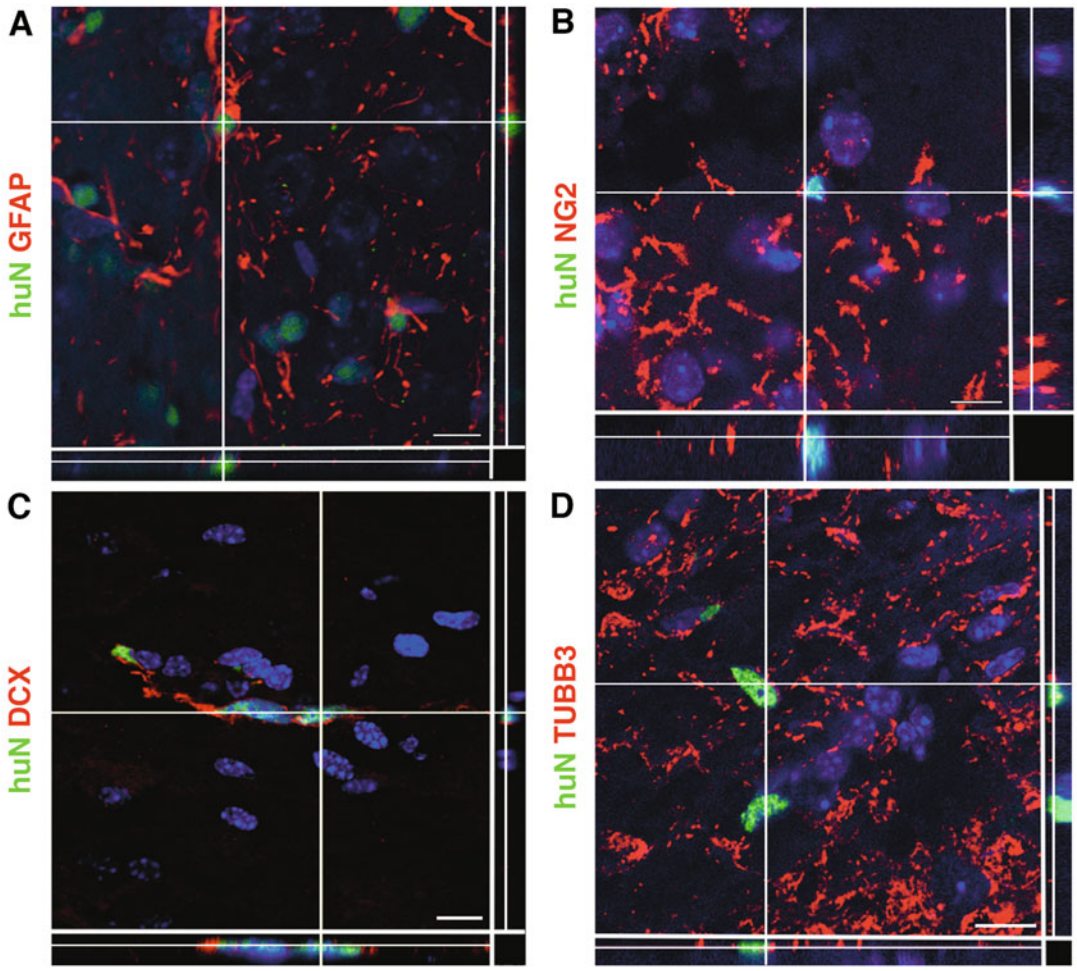

Fig. 1 Multipotency of hNSCs transplanted into the brain of immunodeficient mice. At 6 months upon transplant, hNSCs (huN+ cells) were integrated into the mouse brain and differentiated into astrocytes (**a**, huN+GFAP+), oligodendrocytes (**b**, huN+NG2+), and neurons (**c**, huN+DCX+, and **d**, huN+TUBB3+), thus retaining their multipotency. *Abbreviations*: *huN* human nuclei, *GFAP* glial fibrillary acidic protein, *DCX* doublecortin, *TUBB3* β-tubulin III. Scale bars, 10 μm

Because of all the characteristics above mentioned, raw materials (media, cell culture plastic disposable, etc.) were obtained from GMP-certified suppliers when possible or from suppliers with a quality system verified by the cell factory internal quality assurance manager. All the critical suppliers should be verified by quality unit and periodically inspected.

Human neural stem cells were produced into microbiological and particle-controlled environment (class A surrounded by class B) according to Annex I Vol. 4 European GMP Guidelines. Tissue samples were obtained from screened donors according to European Guidelines on "Certain technical requirements for the donation, procurement and testing of human tissues and cell" (Implementing Directive 2004/23/EC of the European Parliament and of the Council); all the procedures were approved by the competent ethical committees.

2 Materials

2.1 Cell Culture

1. Proliferation medium: 450 ml NeuroCult NS-A Basal Medium (Stemcell Technologies #05750), 50 ml NeuroCult NS-A Proliferation Supplement (Stemcell Technologies #05753), 20 ng/ml human recombinant epidermal growth factor (EGF), 10 ng/ml human recombinant basic fibroblast growth factor (bFGF), 5000 UI/ml heparin, 40 mg/ml gentamicin.
2. Differentiation medium 1: 450 ml NeuroCult NS-A Basal Medium (Stemcell Technologies #05750), 50 ml NeuroCult NS-A Differentiation Supplement (Stemcell Technologies #05754), 10 ng/ml human recombinant basic fibroblast growth factor (bFGF), 5000 UI/ml heparin, 40 mg/ml gentamicin.
3. Differentiation medium 2: 450 ml NeuroCult NS-A Basal Medium (Stemcell Technologies #05750), 50 ml NeuroCult NS-A Differentiation Supplement (Stemcell Technologies #05754), 5000 UI/ml heparin, 40 mg/ml gentamicin, 2% FBS.
4. Heparin (pharma grade).
5. Water for injection.
6. Human recombinant EGF.
7. Human recombinant bFGF.
8. Gentamicin solution (pharma grade).
9. Poly-L-lysine.

10. Cultrex.
11. Fetal bovine serum (FBS).

2.2 Solutions

1. 1× phosphate-buffered saline.
2. 0.4% trypan blue solution.
3. 10 μg/ml KaryoMAX® Colcemid® Solution.
4. Acetic acid.
5. Methanol.
6. Hypotonic solution: 0.075 M potassium chloride solution.
7. G banding solution.
8. Giemsa or Leishman stain at 20% in $Na2HPO_4{\cdot}2H_2O$ KH_2PO_4 pH 6.8 buffer solution.
9. 0.9% NaCl.
10. 1.8% trisodium citrate dihydrate.
11. Tissue wash solution: add 12.5 μl of gentamicin to 10 ml of saline solution.

2.3 Equipment

1. Pasteur glass pipette.
2. Dissecting tools.
3. Scissors, microsurgery clamps.
4. Hemocytometer.
5. 100 mm diameter Petri dish.
6. 15 ml conical tube.
7. 1–30 μl filter tips.
8. 1–200 μl filter tips.
9. 100–1000 μl filter tips.
10. 25 cm^2 flasks with 0.2 μm vented filter cap.
11. 75 cm^2 flasks with 0.2 μm vented filter cap.
12. 10 ml sterile plastic pipettes.
13. 2 ml sterile cryovials.

3 Methods

The following protocol describes the method to isolate, expand, and characterize hNSC obtained from specimens of central nervous system tissue of human fetuses at 8–24 gestational weeks.

3.1 Primary Culture

1. Put the neural tissue into a 100 mm plastic Petri dish prefilled with 10 ml of proliferation medium (at least 1 h at 37 °C, 5% O_2; 5% CO_2).

2. Wash the tissue two or three times in a solution of 0.5 mg/ml gentamicin solution (*see* **Note 1**).
3. Transfer the tissue in a 15 ml tube containing 5 ml of proliferation medium.
4. Dissociate by trituration using a sterile, fire-polished, cotton-plugged glass Pasteur pipette.
5. Let the suspension settle down for 3–4 min.
6. Move the suspension to a clean 15 ml tube, leaving behind the undissociated pieces to the bottom of the tube.
7. Pellet by centrifugation at 110 × *g* for 10 min.
8. Remove the supernatant leaving behind about 160 µl (*see* **Note 2**).
9. Using a 200 µl Pipetman, with the volume set at 120 µl, dissociate the pellet by aspiration/expulsion about 100 times (see **Note 3**).
10. Dilute 10 µl of the sample with trypan blue, and count in hemocytometer according to European Pharmacopoeia "Nucleated cell count" method, adjusting dilution in order to count at least 100 cells in at least three squares.
11. Seed cells at a density of 1 * 10^4 cells/cm^2 in proliferation medium in 25 or 75 cm^2 flask (6 or 12 ml culture media, respectively).
12. Incubate at 37 °C, 5% O_2; 5% CO_2 in a humidified incubator (95% HR) (*see* **Note 4**).
13. Cells should proliferate to form spherical cluster (neurosphere) that should be ready for subculturing (passage) about 7–10 days after plating.
14. Subculturing: transfer the content of the flask in a 15 ml tube.
15. Pellet by centrifugation at 110 × *g* for 10 min.
16. Discard the supernatant leaving behind about 160 µl (*see* **Note 2**).
17. Using a 200 µl Pipetman, with the volume set at 120 µl, dissociate the pellet by aspiration/expulsion about 100 times (*see* **Note 2**).
18. Dilute 10 µl of the sample with trypan blue, and count in a hemocytometer according to European Pharmacopoeia method.
19. Seed and incubate cells as previously described.

3.2 Characterization

3.2.1 Growth Curve

Construction of a growth curve allows you to know the rate of expansion of your cell line:

1. Seed 250,000 cells as previously described in a 25 cm^2 and keep culturing them for at least five passages.
2. At each passage count the obtained cells and seed again at the same density.
3. Create a chart representing the logarithmic number of viable cells of each passage, versus the day in vitro since the beginning of the experiment.

3.2.2 Karyotype Analysis

1. Plate 300,000 cells in a 25 cm^2 flask containing 6 ml of preconditioned medium.
2. Incubate 37 °C, 5% O_2; 5% CO_2 for 48–120 h.
3. Add 30 μl of Colcemid solution (1:200) and incubate for 3 h.
4. Transfer cell suspension in a 15 ml tube.
5. Pellet by centrifugation at 110 × *g* for 10 min.
6. Discard the medium carefully leaving about 200 μl.
7. Suspend the pellet using a 200 μl Pipetman.
8. Add 10 ml of hypotonic solution and leave the solution at 37 °C for 30 min.
9. Centrifuge at 110 × *g* for 10 min.
10. Discard the solution and add the fixative (3:1 methanol/acetic acid), and leave 10 min at room temperature.
11. Repeat three times the last two operations.
12. Proceed with standard Giemsa chromosome staining (if you need you can store the cell suspension in your fridge up to 5 days before staining).
13. Apply two or three drops of metaphase suspension on your slide.
14. Put the slide over a water bath (37 °C) for 1–2 min.
15. Check under the microscope for the presence of good metaphase spread, if necessary centrifugate again your sample at 110 × *g* for 10 min and discard part of the supernatant, and then prepare another slide.
16. Let the slide dry in incubator overnight.
17. Put in a trisodium citrate/physiological solution (1:1) for 5 min, and then wash in bi-distilled water and let dry.
18. Put in a 0.02% trypsin solution in distilled water for 10–30 s.
19. Wash in physiological solution.
20. Stain with 20% Giemsa solution for 1–10 min.
21. Wash with water and let dry at room temperature.

3.2.3 Differentiation

1. Carefully, place a round-glass coverslip in each well of a 12-well plate.
2. Cover with about 300 μl of Cultrex solution, and incubate for about 1 h at 37 °C.
3. Discard the supernatant.
4. Seed 7–10 * 10^4 cells in each well, in differentiation medium 1; incubate for 3 days.
5. Replace medium with differentiation medium 2; incubate for 7 days. At day 4, replace half of the media, carefully avoiding to touch the gel at the bottom of the well.
6. Remove glasses from the wells and put them in another plate, and proceed with fixation using standard protocols for immunocytochemistry (ICC). Once fixed, samples can be stored up to a month before analysis.
7. For the staining you can use custom-made protocols or commercially available kit, depending on the nature and amount of markers you want to detect in your sample. *See* Vescovi et al. [6] for an example of staining protocol.

3.2.4 Clonal Efficiency

Clonal efficiency assay shows the percentage of plated cells that retains the ability to form clonal neurospheres under stringent culture conditions:

1. Coat six wells of a flat bottom, multi-well plate with 1 ml of poly-L-lysine solution.
2. Incubate for 1 h at 5%O_2 5% CO_2 and 37 °C.
3. Remove supernatant.
4. Seed cells at a density of 250 viable cells/cm^2 in proliferation medium. Incubate for 7 days as previously described.
5. Using an inverted microscope equipped with a grid ocular, count the number of neurospheres that measure 50 and 100 μm in diameter.
6. Calculate the clonal efficiency, using the ratio between the number of neurospheres obtained and total cell seeded.

4 Notes

1. The kind of antibiotics you use and the amount depends on the final use of your product. If you are working in a R&D laboratory, you can also add different antibiotics for the entire culture. For cell therapy products, antibiotics should be avoided to reduce the risk of possible adverse event after the product transplant.

2. When the cell pellet is visibly small, less than 160 μl medium should be left in the tube. A too high medium volume does not permit an efficient dissociation of neurospheres.
3. The dissociation of pellets is the crucial step in subculturing stem cells. In order to verify the efficient dissociation of the cells, let a small drop of cell suspension glide down the wall of the tube. If cell clusters are visible, continue pipetting up and down. If neurospheres are not completely disassociated, in subsequent passages cells could form aggregates instead of neurospheres and finally differentiate in astrocytes, neurons, or oligodendrocytes. However, avoid to pipet more than necessary, as that could induce cell death or cell differentiation, as well.
4. If the cells begin to grow in adherence, the humidity level of the incubator must be checked. A humidity rate too low could induce cells to grow in adherence.

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