Chapter 4
Neural Differentiation of Rodent Neural Progenitor Cells and Isolation and Enrichment of Human Neural Progenitor/Stem Cells

Antonio H. Martins, Jose L. Roig-Lopez, and Maxine Nicole Gonzalez

Abstract Neural progenitor cells (NPC) are multipotent and give rise to neurons, astrocytes and oligodendrocytes. NPC possess the ability of *in vivo* and *in vitro* proliferation and can therefore be expanded for research proposes. Cell proliferation generates a three dimensional aggregate called neurospheres; posteriorly, these neurospheres can be differentiated into multiple cell types and become an important tool for understanding the mechanisms regarding cell differentiation and neuronal regeneration. In this chapter, we describe the extraction of rat neural progenitor cells from embryonic telencephalon (14 days) and generation of neurospheres. Next, we describe protocols to isolate and proliferate human fetal stem cells derived from elective abortion, recognizing the ethical dilemmas. The advantage of using the neurospheres-rat model is that the differentiation can be achieved by removing the growth factors and allowing cell differentiation in poly-D-lysine/laminin coated plates. The cells can then be dissociated and grafted in animals or be differentiated and grafted, depending on the research hypothesis. The endogenous cells can be followed by staining with lipophilic dyes or analyzed by adding 5-Bromo-2′-deoxyuridine (BrdU) using the posterior double immunofluorescence technique, which identifies the post-mitotic neural progenitor cells and the fate acquired. The methods here described will help the researcher to perform cell extraction, differentiation without cell enrichment, track the proliferating neural progenitor cells and perform characterization after the cell graft, as well as isolate and maintain human fetal stem cells.

Keywords Neurosphere • Differentiation • Rat • Human

A.H. Martins (✉)
Pharmacology and Toxicology Department, School of Medicine
University of Puerto Rico Medical Science Campus, San Juan, PR, USA

Department of Biochemistry, Universidad Central del Caribe, Bayamón, PR, USA
e-mail: antonio.martins@upr.edu

J.L. Roig-Lopez
School of Science and Technology, Universidad Del Este, AGMUS, Carolina, PR, USA

M.N. Gonzalez
Department of Biochemistry, Universidad Central del Caribe, Bayamón, PR, USA

H. Ulrich, P. Davidson Negraes, *Working with Stem Cells*,
DOI 10.1007/978-3-319-30582-0_4

4.1 Introduction

The notion that the central nervous system (CNS) was unable to produce new neurons was once held certain by the scientific community. Discoveries over the past 20 years of neuronal progenitor cells (NPC) and neural stem cells (NSC) in adult animals have caused euphoria, bringing the possibility to repopulate the brain with new neurons capable of reestablishing connections, either physiologically lost with age or due to a variety of pathologic conditions such as traumatic brain injury, stroke, Alzheimer and amyotrophic lateral sclerosis (Einstein and Ben-Hur 2008). NPCs are defined as multipotent and self-renewing, give rise to neurons, oligodendrocytes, and astrocytes without generating other cells from embryonic layers other than the ectotherm, thereby showing more restriction. In other words, NPC are less committed than NSC and exist in low amounts in the CNS.

Neural progenitor cells are basically localized in thee niches: the olfactory bulb, the subventricular zone (SVZ), and the subgranular zone of dentate gyrus (SGZ). These niches are distinct regarding the ability to respond to different stimuli or in the ability to produce a specific type of neuron. For example, several groups have demonstrated that the adult human NPCs at SVZ are the primary site of proliferation and neurogenesis in patients with neurodegenerative diseases (Nait-Oumesmar et al. 2007). At the same time, NPC derived from dentate gyrus is more suitable to proliferation regarding memory formation (Eckenhoff and Rakic 1988). To study differentiation processes, NPC derived from the embryonic brain from day 14 was used in several works (for review see Trujillo et al. 2009). However, even with a great capability for proliferation, the total number of NPC remains scarce. To increase the total number of cells for cell graft or to study the process of differentiation, the NPC can be cultured *in vitro* in tridimensional agglomerates, called neurospheres, in the presence of epidermal growth factor (EGF) and fibroblast growth factor (FGF). The easy induction of differentiation by removing the growth factors and the ability to drive the cell differentiation into neurons (using retinoic acid), oligodendrocytes (with insulin-like growth factor 1, IGF-1), and astrocytes (using bone morphogenetic protein), make NPC important in the fields of both differentiation and neurorepair.

Here, we will focus on the extraction, proliferation, maintenance and analyses of cell differentiation by cell markers, using immunofluorescence and flow cytometry for neurospheres from rats and mice. In this chapter, we also describe human NPC purification and expansion.

4.2 Protocols

4.2.1 Preparation of Non-adherent Cell Culture Flasks

1. Use non-adherent culture flasks from brands of your preference or,
2. Prepare an anti-adherent solution of 12 % of 2-hydroxyethyl methacrylate MW 20,000 (6 g of 2-hydroxyethyl methacrylate (Sigma-Aldrich, Cat. No. 128635)

in 50 ml of 95 % ethanol). Because it is difficult to dissolve, keep it covered in shacker at 37 °C overnight;
3. Cover the flask bottom with the solution, remove the excess (which can be re-used) and leave the flask open inside the cell culture hood to completely dry;
4. After it is dry, wash three times with phosphate-buffered saline (PBS). It is then ready to use.

Note: *Use sterilized beaker, spatula, and so on to prepare the solution even if 2-hydroxyethyl methacrylate is not sterile. This will decrease the chances of contamination by reducing the number of bacterial or fungi spores. Remember that ultraviolet light does not penetrate plastic or glass. The flasks can be stored closed for several months at room temperature.*

4.2.2 Animals

1. Time-pregnant rats (for instance: Wistar, Sprague Dawley with 14–16 weeks of age) with 14.5 days of pregnancy purchased from the company of your choice or, paired one male and one female for approximately 12 h; after this period of time perform a vaginal smear using distillated water. *Do not use PBS or other buffer because of drying and crystal formation. Use a wet swab and a clean slide; this procedure can be performed in rats but not in mice, because in the latter you will remove the vaginal plug.*
2. Introduce a wet swab in the vagina and then make a smear in a clean slide. The presence of spermatozoids is a potential sign that the rat will become pregnant. Sometimes, despite the presence of spermatozoids, the rat may not be pregnant. *Non-observance also does not rule out the possibility of pregnancy.* We roughly calculate an 80 % chance of pregnancy.

4.2.3 Surgery (Instruments and Materials)

1. Sterilize the instruments in the autoclave using a steel box or an instant sealing sterilization pouch.

For removing the head

2. One operating scissor 5.5″ (Roboz Company, Cat. No. RS-6812)—For cutting the rat's head;

For holding the skin of the rat while the skull is removed

3. Curved forceps 7″ (Roboz Company, Cat. No. RS-5271)

For meninges peeling

4. Two micro-dissecting forceps 4″ slightly curved 0.8 (Roboz Company; Catalog number RS-5135)—For placing the cortices in the petri dishes containing medium, four micro-dissecting tweezers (Roboz Company, Cat. No. RS-4972);

For separating the cortices

5. Two Micro-dissecting forceps 4″ angled fine sharp (Roboz Company, Cat. No. RS 5095).

4.2.4 Culture Materials

- Serological pipettes 5, 10, and 25 ml.
- Thee 60 mm petri dishes.
- Sterile syringe of 5 ml.
- Invert phase microscopy.
- Different micropipette tips (0.2 and 1 ml).
- Cell strainer 40 μm BD Falcon.
- Laminin.
- Centrifuge conical tubes of 15 and 50 ml.
- Stomacher 80 Seward® Lab System.
- Model 80 Bags for Stomacher.
- Trypsin/EDTA.
- Fibroblast growth factor.
- Epidermal growth factor.
- Poly-D-lysine.
- Heparin.
- Dulbecco's Modified Eagle's Medium (DMEM) and F12 medium.
- Fetal bovine serum (FBS).
- B27 supplements 50× Gibco Life technologies (Cat. No. 12587-010).
- Trypan blue solution (for measurement of cell viability).

4.3 Methods

4.3.1 Neurospheres from Rat Embryonic Telencephalon

My collaborators and I have used for several years the protocol described below. We developed it to obtain NPCs and to generate neurospheres from mice and rats. Although the cells can be placed in any non-adherent cell culture flasks or dishes after extraction, we recommend flasks, which decreases the chances of contamination. After distributing the cells, carefully look inside the flasks to find any

contaminants such as fungi or bacteria. It is not necessary to anesthetize the fetuses for the surgery since at this stage of development they do not register pain and the head will be taken out following the NIH guidelines for euthanasia.

1. Use time-pregnant rats (14.5 days or mice of 12.5 days);
2. There are several protocols for animal preparation for the surgery; (a) euthanize using an overdose of Isoflurane (5 % until the rat stop breathing) or (b) CO_2 chamber;

Note: *Use a specific set of instruments for opening the skin and another set to remove the uterus containing the fetuses. This practice is important to avoid contamination. Remember that the skin is disinfected but not sterilized.*

3. Perform an antiseptic cleaning of the skin using ethanol 70 % or iodine solution (10 % povidone-iodine);
4. Open the skin using scissors and forceps;
5. Remove the uterus using a different forceps and put it in 60 mm petri dishes;
6. In another plate pour 5 ml of DMEM/F12 70 %/30 % medium;
7. Open the uterus and let the fetuses felt in the liquid;
8. Grasp the fetuses and decapitate those (RS 6812);
9. Pour 5 ml of medium in a second petri dish;
10. Place the head in the petri dish and discard the body in a plastic bag;
11. Using two micro-dissecting tweezers (RS 5882), sustain the animals and make an incision between the eyes;
12. Then, go laterally with the tweezers as follows: one blade of the tweezer will be inside the skin and the other, outside. Using the tweezer lift and remove the portion that will be the future skull;
13. Remove the meninges completely, then the cerebellum and everything from underneath each telencephalon;
14. Pour 2 ml of medium inside the Model 80 bags for Stomacher 80, leaving space to seal;
15. Place the bag into the Stomacher 80 and close the door. The cell dissociation is performed during 2.5 min at high speed or, gently triturate the tissue using trypsin/EDTA through different series of descending micropipette tips to make single cell suspension. Add FBS to inactivate the trypsin;
16. Filter the bag content using a 40 μm cell strainer coupled to a sterile syringe;
17. The volume should be dispensed into a 15 ml conical tube;
18. Centrifuge for 5 min at 200 × g, add 10 ml of fresh medium and centrifuge again;
19. Repeat Step 18 twice.

4.3.2 Neurosphere Formation

At this point, there are mixtures of cells smaller than 40 μm. The following protocol is used to expand the neural progenitor cells in the neurosphere shape.

Prepare the Plates for Plating Neurospheres

1. Count the cells using Trypan-blue and add 180,000–200,000 cells/ml of viable cells into the anti-adherent flasks or into coated flasks using the following medium: DMEM/F12 70 %/30 %, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, 2 mM L-glutamine 20 ng/ml fibroblast growth factor, 20 ng/ml epidermal growth factor, 5 μg/ml of heparin (Sigma-Aldrich, Cat. No. 1304005), 2 % B27 at 37 °C in 5 % CO_2;
2. Allow the cells to grow for 10 days changing half of the medium every 3–4 days.

4.3.2.1 Neurosphere Phenotype Analysis

After 10 days, the neurospheres can be analyzed. Due to variation in size, we recommend to use neurospheres with similar diameter to avoid structural differences. For example, large neurospheres possess more cell death in the center.

Examples of Analysis

A. Reverse Transcription-polymerase chain reaction (RT-PCR) to analyze gene expression;
B. Western blot to evaluate protein expression;
C. Flow cytometry analysis to quantify the number of neural progenitor cells and a specific protein of interest;
D. Immunofluorescence to analyze protein expression and localization.

Neurosphere Immunofluorescence

Preparation

1. Collect neurosphere samples of similar size;
2. Wash twice to remove the excess medium;
3. Fix using 4 % paraformaldehyde (PFA 4 %) at room temperature (25 °C) for 1 h;
4. Afterwards, wash thee times with PBS and then transfer the neurospheres to a solution of PBS/sucrose 10 % (w/v) for 1 h, followed by PBS/sucrose 20 % (w/v) for 1 h and finally PBS/sucrose 30 % (w/v) for 16–18 h at 4 °C;
5. Put the neurospheres in HistoPrep (Fisher Scientific, Cat. No. SH75-125D) and freeze in dry ice for 5 min, followed by freezing at −80 °C overnight;

Note: *Place the neurospheres at the bottom and very carefully add the HistoPrep, in order to keep the neurospheres at the same plane.*

6. Make slices of 10 μm using a Cryostat and put them onto signalized Superfrost slides (Fisher Scientific, Cat. No. 48311-703).

Proceed for immunofluorescence detection

There are different antibody dilutions and different blockers that could be used, depending on the protein of interest. Nonetheless, we will give a general protocol for permeabilization and blocking to analyze nestin, glial fibraly acidic protein (GFAP) and β3-tubulin expression patterns.

7. Prepare a permeabilization and blocking solution using 0.1 % Triton X-100 (Sigma Aldrich, Cat. No. X100) and 3 % fetal bovine serum in PBS; incubate the neurospheres at 25 °C for 30 min;
8. Add the primary antibody (1:250) using PBS and 3 % of fetal bovine serum and incubate for 2 h at 25 °C;
9. Wash thee times with PBS;
10. Add the secondary antibody (1:250) and incubate for 2 h at 25 °C. *Remember to use antibodies raised in different species in case of double staining*;
11. Wash three times with PBS;
12. Place a mounting medium for immunofluorescence and add a coverslip.

4.3.2.2 Neurosphere Differentiation

Prepare dishes or 6-well plates with poly-D-lysine (Sigma-Aldrich, Cat. No. P7280) and laminin (Sigma Aldrich, Cat. No. L4544), as following:

i. Prepare a solution of 0.05 mg/ml of poly-D-lysine, MW 30–70 K, and then add 0.5 ml/well 16–18 h (6-well plate). Remove the poly-D-lysine and wash the wells with sterile distilled H_2O twice. Let it dry for 1 h.
ii. Prepare a laminin solution and use it at final concentration of 20 μg/ml in water. Add 200 μl into the previously coated poly-D-lysine dishes making circles using a pipette (recommended concentration by Sigma-Aldrich is 1–2 μg/cm^2). The circles will decrease the amount of volume used in each dish. Do not allow the laminin to drain to the edges. Incubate the dishes or 6-well plates at 37 °C for 1 h. Wash twice using cell culture medium. We recommend not to store the plates coated with laminin.

1. Allow the neurospheres to decant and transfer to a 15 ml conical centrifuge tube; wash the neurospheres three times with 10 ml of medium (70 %/30 % DMEM/F12) to remove any trace of growth factors;
2. Place the neurospheres in the dishes or 6-well plates prepared with poly-D-lysine and laminin;
3. Let them to differentiate for 7 days. A radial pattern of cell differentiation can be visualized; next, analyze the protein of interest.

4.3.3 Characterization of the Cells Obtained Using the Described Protocol

To verify proper cell differentiation, we recommend analysis of the cells using immunofluorescence and/or flow cytometry. After differentiation, the cells should express β3-tubulin, GFAP, S100β, and decreased expression of nestin (Martins et al. 2008).

4.3.3.1 For Immunofluorescence

1. Fix the differentiated neurospheres using 4 % paraformaldehyde at 4 °C for 30 min. Wash the cells twice using PBS for 5 min;
2. Permeabilize and block the cells using a solution of 0.1 % Triton and 10 % goat serum in PBS at 25 °C for 30 min;
3. Add the primary antibody (1:750 anti-GFAP Cy3, Sigma-Aldrich, Cat. No. G3893 or 1:1000 anti-β3-tubulin (Abcam, Cat. No. 18207) for 2 h at 25 °C using PBS and 1 % of goat serum), or at 4 °C overnight;
4. Wash thee times with PBS (5 min each);
5. Add the secondary antibody (1:1000) at 25 °C for 1 h;
6. Wash thee times with PBS (5 min each);
7. Add 4′:6-Diamidino-2-Phenylindole; dihydrochloride- (DAPI) (Life Technologies, Cat no. D1306) solution to counterstain the nuclei;
8. Put a mounting medium for immunofluorescence and add a coverslip. Observe under a inverted immunofluorescence microscope.

Note: *Remember to use primary antibodies raised in different species and secondary with different colors in case of double staining.*

Several different markers are listed in Table 4.1. Some cellular markers are shared by human stem/progenitor cells and neuron and glial cells, listed in Table 4.2.

4.3.3.2 Flow Cytometry

1. Dissociate the neurospheres with Accutase (Sigma-Aldrich, Cat. No. A6964) or trypsin into single cells using different decreasing micropipette tip size. When using trypsin, inactivate the trypsin with 10 % fetal bovine serum (final concentration) (BioAnalytical Instruments, Cat. No. 16000-044);
2. Centrifuge at 200 × *g* for 5 min;
3. Fix the cells in ice-cold 1 % PFA in PBS for 20 min, then wash with PBS;
4. Incubate the cells using 2 % of fetal bovine serum in PBS and 0.1 % Triton for 30 min;

Table 4.1 Neural progenitor/stem cells markers during development and *in vitro* differentiation

Target	Markers	Reference
Neural stem cells	CXCR4	Itoh et al. (2009)
	FABP7/B-FABP	Yun et al. (2012)
	ID2	Park et al. (2013)
	Musashi-1	Kirik et al. (2013)
	Nestin	Park et al. (2010)
	Notch-1	Nagato et al. (2005)
	SOX-1	Venere et al. (2012)
	SOX-2	Ellis et al. (2004)
	SSEA-1	Son et al. (2009)
Neural progenitor cells	Activin A	Rodriguez-Martinez et al. (2012)
	FABP7/B-FABP	Arai et al. (2005)
	GFAP	Liu et al. (2010)
	NCAM-1/CD56	Marmur et al. (1998)
	Nestin	Suzuki et al. (2010)
	ROR2	Endo et al. (2012)
	SOX-2	Graham et al. (2003)
	Vimentin	Kim et al. (2009)
Glial progenitor cells	Nestin	Messam et al. (2000)
	S100B	Raponi et al. (2007)
Young neurons	β3-Tubulin	Braun et al. (2002)
	Calretinin	Brandt et al. (2003)
	MAP2	Arai et al. (2005)
	NCAM-1/CD56	Terkelsen et al. (1992)
Mature neurons	Calbindin	Young et al. (2000)
	ID2	Neuman et al. (1993)
	NeuN	Seki (2002)
	Synaptophysin	Gingras et al. (2007)
Oligodendrocyte-like cells	Olig1	Ohnishi et al. (2003)
	O4	Lu et al. (2000)
Astrocyte-like cells	EAAT1/GLAST-1	Miralles et al. (2001)
	GFAP	Brahmachari et al. (2006)

5. Incubate with primary antibodies specific for neurons (β3-tubulin, Abcam, Cat. No. 18207), astrocytes (1:500 anti-GFAP Cy3 Sigma-Aldrich, Cat. No. G3893) and mature neurons microtubule associated protein 2 (1:500 MAP-2, Cell Signaling Technology, Cat. No. 4542) for 2 h;
6. After 2 h, wash twice with PBS, and then incubate the cells with 1:500 Alexa Fluor 488- or 555-conjugated secondary antibodies (Life Technologies and Molecular Probes, Cat. No. A11001 and A21428, respectively);

Table 4.2 Human neural progenitor/stem cells markers during development and *in vitro* differentiation

Target	Markers (human)	Reference
Neural stem cells/neural progenitor cell	$CD133^+$ (Prominin)	Uchida et al. (2000)
	$CD34^-$	
	$CD45^-$	
	Nestin	Tohyama et al. (1992)
	GFAP	
	Integrin subunit α6	Hall et al. (2006)
	Integrin subunit β1	
	Pax-6	Hansen et al. (2010)
	SOX-2	
	TRBR2 (EOMES)	
	Musashi-1	Dhara et al. (2008)
Glial progenitor cells	Nestin	Hansen et al. (2010)
	CD133 radial glia	Pfenninger et al. (2007)
Young neurons	β-3Tubulin	Liu et al. (2010)
	TBR2 (EOMES)	Hansen et al. (2010)
	Calbindin	Eriksson et al. (1998)
Mature neurons	MAP2	Anacker et al. (2011)
	Synaptophysin	Gingras et al. (2007)

7. Analyze in flow cytometer (Fc500, Beckman Coulter, Fullerton, CA) using an argon laser line for fluorescence excitation (FL1 525 nm and FL2 575 nm, band pass filter). Between 10,000 and 50,000 events per sample should be acquired with fluorescence measured in logarithmic scales. Set up the background fluorescence using unlabeled cells and those labeled with secondary antibody alone. Use it to set gating parameters between antigen-positive and negative cell populations;
8. Use forward and side light-scatter gates for exclusion of cell aggregates and small debris;
9. Use Cyflogic software (http://www.cyflogic.com) and plot the results in a histogram format.

4.4 Use of Neural Progenitor Cells After Stroke

The majority of cerebral strokes are caused by the occlusion of a blood vessel in the brain, which decreases oxygen supply to the affected area. An early onset neuronal death begins within a minute after the beginning of an ischemic stroke; it occurs in the area most affected by the ischemia. This area, called the infarct core, has either a low or no blood flow. The resulting oxygen and glucose deprivation is followed by a decrease in ATP, thus triggering an excitotoxicity cascade. The

energy failure stops the sodium and potassium pumps and promotes collapse of the neuronal transmembrane ionic gradient, Ca^{2+} influx and release of glutamate, which causes excitotoxicity (Barone et al. 1997; Dirnagl et al. 1999). The ischemic brain injury also increases the synthesis of inflammatory cytokines that spread the inflammation into the surrounding area (Huang et al. 2006; Iadecola and Alexander 2001). The brain tissue that surrounds the core is called the penumbra and can be saved if neuroprotective drugs or thombolytic drugs are administrated timely. The penumbra neurons can either recover and survive, or be recruited to the ischemic core and die. The period during which the penumbra neurons can be salvaged is called "the therapeutic window of opportunity". Therefore, the neuroprotective interventions that target delayed processes such as apoptosis and inflammation in the penumbra have a potential for clinical treatment in acute stroke (Xu et al. 2004).

Interestingly, recent evidence suggests that global and focal ischemia enhance the proliferation and differentiation into neurons in several zones including dentate gyrus, the anterior subventricular zone, and the posterior periventricular zone, adjacent to the hippocampus. Moreover, Arvidsson et al. (2002) showed that the initial generation of neuroblasts following a stroke, far exceeds the final number of surviving new neurons, showing that number could be increased. The plasticity could allow brain repair without the need of cell graft (Andres et al. 2011) and much evidence exists describing the involvement of NPCs in stroke treatment (Andres et al. 2011; for review see Barkho and Zhao 2011). Grafted NPCs are "known to have the ability of migrating long distances to damage sites after brain injury and differentiate into new neurons" (for a review see Barkho and Zhao 2011).

4.4.1 Protocol to Observe Cell Migration/Proliferation After Stroke

4.4.1.1 Middle Cerebral Artery Occlusion (MCAO)

Surgical procedures should be performed using sterile/aseptic techniques in accordance with institutional guidelines and the initial assistance of the veterinarian. Sprague-Dawley rats weighing 250–300 g will fast for no more than 4 h to avoid hyperglycemia after administration of nitric oxide and isoflurane. The animals will be subjected to permanent or transient MCAO as previously described (Xu et al. 2004; Martins et al. 2015).

Middle Cerebral Artery Tools

- Rodent Brain Matrices (Stoelting, Cat. No. 51388).
- 2 U Forceps (Stoelting, Cat. No. 52110-31).
- Silk Sutures (4-0 & 6-0) (Ashaway).

- 2 U Microvascular Clamp (Fine Science Tools, Cat. No. 18055-040).
- Cautery/Bipolar coagulation Forceps.
- Micro-Serrafine Clamp applicator (Fine Science Tools, Cat. No. 18056-14).
- Dual Gooseneck Illuminator (Stoelting, Cat. No. 59259).
- Anesthesia system (Stoelting).
- Micro-dissecting Forceps (Stoelting, Cat. No. 52102-54 and 52102-35).
- Anesthesia scavenger (Stoelting).
- Spring Scissors (Stoelting, Cat. No. 52130-00).
- Micro-dissecting scissors (Stoelting, Cat. No. 52132-42).
- Needle Holder (Stoenting. Cat. No. 52181-84).
- Heating Pad.
- Shaver.
- Surgery Microscope.
- Cotton tipped applicators.

To observe endogenous neural progenitor cell proliferation after stroke

The NPCs could be analyzed by using a pulse or a cumulative protocol of 5-bromo-2′-deoxyuridine (BrdU), a thymidine analogue (Sigma-Aldrich, Cat. No. B5002) diluted in 0.9 % saline, depending on the researcher interest.

1. As an example, inject 50 mg/kg of BrdU intraperitoneal every 4 h for 12 h in the same day of the animal sacrifice (Zhang et al. 2001) to obtain a temporal profile;
2. To observe all proliferating NPCs, inject intraperitoneal 50 mg/kg of BrdU, saline immediately after the surgery. Inject 50 mg/kg every 24 h until euthanasia;
3. Remove and freeze the brain using HistoPrep;
4. Cut the brain into 40 μm slices and put them into Superfrost slides;
5. Fix the slices using 4 % of paraformaldehyde for 30 min, then wash using PBS;
6. Prepare a permeabilization and blocking solution using 0.1 % Triton, 5 % FBS or BSA in PBS for 10 min; add the primary antibodies in 1 % FBS in PBS at room temperature for 16–18 h; wash twice with PBS;
7. Add the secondary antibodies: for instance, anti-mouse Alexa-Fluor 488 and anti-rabbit Alexa-Fluor 555 for 2 h.

Note: *The primary antibodies should be raised in different species in order to make a double staining. We recommend using Anti-BrdU plus a different combination of antibodies to detect newly generated neural progenitor cells that differentiate into astrocyte, (BrdU*$^{+}$*GFAP*$^{+}$ *cells), mature neurons (BrdU*$^{+}$*NeuN*$^{+}$ *cells) or oligodendrocyte (BrdU*$^{+}$ *Anti-oligodendrocyte specific protein (Abcam, Cat. No. ab53041) positive cells), migrating newly neural progenitor cells or migrating newly immature neurons (BrdU*$^{+}$ *Doublecotin DCX*$^{+}$*, Abcam, Cat. No. ab18723).*

4.4.1.2 Injection of Neural Progenitor Cells

Prepare a stock solution of lipophilic $DiOC_{18}(3)$ in dimethyl sulfoxide (DMSO) at 1 mg/ml.

Note: *Dimethylformamide (DMF) is the solvent indicated by the company, however our laboratory dilutes in DMSO without complications.*

1. Prepare the NPCs as described above;
2. Stain the cells using a lipophilic dye 3,3′dioctadecyloxacarbocyanine perchlorate $DiOC_{18}(3)$ (ThermoFischer Scientific, Cat. No. D-275) as following;
3. Prepare 2.5×10^6 of dissociated NPCs; pass the cells through a 40 μm cell strainer to avoid cell clamps and put them in complete NPC medium;
4. Add 5 μl of a stock solution of 2.0 mg/ml to the cells and incubate at 37 °C for 1 h;
5. Wash the cells twice using fresh medium;
6. Check under fluorescence microscope the quality of the staining (intensity and if all cells are stained);
7. Graft of 120,000 cells/2 μl will be injected by means of the cisterna magna (intracranial) using a glass micropipette or a 1 cc insulin syringe, as previously described by Mitome et al. (2001);
8. After a period of time, which will vary according to the research interest, remove the brain and perform a slice preparation as described above;
9. Chcek the fluorescence under an immunofluorescence microscope;
10. The researcher has also the possibility to verify the type of cell that was grafted and posteriorly differentiated;
11. Use antibodies, such as anti-NeuN (Abcam, Cat. No. ab104225) to detect the differentiation of NPCs into neurons, anti-GFAP for astrocytes, and anti-Nestin (Abcam, Cat. No. ab6142) for undifferentiated cells. Remember to use different colors for staining cells; for instance use DiO and DiD (Molecular Probes) and distinct antibodies.

4.5 Human Fetal Tissue as Source of Neural Stem Cells/ Neural Progenitor Cells

Fetal samples for enrichment and isolation of NSCs are usually discarded as biohazard samples from elective abortions performed in the USA and other countries. To properly obtain these samples, researchers must consult with an institutional biosafety and compliance officer to develop an Institutional Review Board (IRB) Protocol at least 6 months prior to experimental procedures. Other options to acquire tissue samples depend on research centers and/or non-profit organizations. For CNS samples, it will usually be crude brain tissue from the end of the first to the second trimester. Despite multiple moral and ethical issues, established protocols make this source of human neural stem cells very attractive.

Fetal human adult or somatic neural stem cells come from mitotically active regions of the CNS during development, after 5 weeks of gestation, to adult human regions of the CNS such SVZ and outer SVZ (OSVZ) along the ventricles. These NSCs share the basic characteristic of self-renovation and the capacity, upon differentiation, to give rise to neurons, glia and oligodendrocytes (Vescovi et al. 1999). At 10.5 weeks of gestation from a diencephalon sample, 1 % of 1000 cells form neurospheres using the regular serum free media with EGF/FGF-2 and supplemental hormones/salt mix. These NSCs, shaped as neurospheres, are multipotent upon differentiation, which involves the removal of growth factors (EGF/FGF-2). The cells will attach to the substrate via the poly-ornithine coating. Such cultures can be maintained up to 2 years with a subculturing or passage rate every 2 weeks. Finally, and more importantly, differentiated neurons and glial cells survive the transplantation procedure into the striatum of immunosuppressed rats.

The nature of the human neural stem cells was discovered by enrichment, using cell sorting of human fetal samples with antibodies previously used to isolated human Mesenchymal/Hematopoietic Stem cells. The NSC in fetal samples was found to be CD133+ (Prominin), CD34−, and CD45−. Single cells with these lineage markers form a neurosphere under standard proliferative conditions (Uchida et al. 2000; Hall et al. 2006). It was later established that CD133 stains the radial glial/ependymal cell type at the early postnatal stage, and ependymal cells in the adult brain, but not neurogenic astrocytes in the adult human SVZ (Pfenninger et al. 2007). In another work by Hall et al. (2006), human fetal tissue (8–10 weeks of gestation) enriched by the use of integrin subunits α6 and β1, was capable to form NSC cultures. The presence of others markers of NSCs, such as Sox2, Sox3, Nestin, Bmi1 and Musashi1 (Sun et al. 2009) were found to corroborated the presence of such cells.

The developing fetal human forebrain has a massively expanded outer region throught to contribute to cortical size and complexity of the prospective human cortex. However, SVZ cytoarchitecture was presumed to be similar to rodent species and progenitor cell types; its contribution to neurogenesis is still not well understood. A meticulous analysis showed that large numbers of radial glia-like cells and intermediate progenitor cells populate the human fetal SVZ (Hansen et al. 2010). A second population of radial glia-like cells, named OSVZ, was found to have long basal process but, surprisingly, are non-epithelial as they lack contact with the ventricular surface. By real-time imaging, clonal analysis with mitotic markers and using viral markers, the Krigelstein group (Hansen et al. 2010) showed that these cells can undergo proliferative divisions and self-renewing asymmetric divisions to generate NPCs that can further proliferate. This OSVZ with their population of outer Radial Glia (oRG) may be isolated from human fetal samples and expanded in cultures due to the fact that the oRG are more numerous and prominent at the second trimester of gestational week of the fetus.

4.5.1 Isolation, Enrichment and Culture of Human Fetal Neurospheres

Fetal discarded tissue from elective abortions can be obtained from Advanced Bioscience Resources, a nonprofit organization that collects and sends human fetal tissue for clinical and biomedical purposes, and follows all state and federal regulatory procedures. Under the laws of the US Commonwealth of Puerto Rico, fetal tissue is classified as a cadaveric donation. In our experiments we used complete brain samples from 16 to 18 weeks of gestation. Every order required proper clearance and approbation of IRB and Bio-Safety Institutional compliance.

Materials

- Accutase 1× (Sigma-Aldrich, Cat. No. 6964).
- B-27 supplement. **No vitamin A**. (Cat. No. 12587-010) *Note: contains insulin, antioxidants cocktail among others hormones.*
- Micropipette P1000, Blue Tips, sterile (Fisher Scientific, Cat. No. 02-707-405).
- DMEM/F12 1:1 (Sigma-Aldrich, Cat. No. D8437).
- Sterile 10 ml 13-678-11E, Tubes 50 ml Falcon (Fisher Scientific, Cat. No. 352098).
- GlutaMax (Invitrogen-Life Technologies, Cat. No. 35050061).
- 6-Well Plate Falcon (Fisher Scientific, Cat. No. 353046).
- Leukemia Inhibitory Factor, final concentration 5 ng/ml. (Peprotech, Cat. No. 300-05).
- Recombinant Human EGF (Catalog number AF 100-15), final concentration 10 ng/ml.
- Neurospheres Proliferation Media (NPM): D-MEM/F12 1:1, EGF, FGF-basic, LIF, Glutamax, antibiotic-antimycotic, and B-27 supplement.
- Recombinant Human FGF-basic (154 a.a.) (Catalog No 100-18B) final 10 ng/ml.
- Antibiotic-antimycotic 100×. (Gibco, Cat. No. 15240-062).

To isolated and enrich Neurospheres from tissue

1. Decant shipping media, put tissue in sterile cold basal media with 2× antibiotic-antimycotic and a mix of wide spectrum antibiotics in a 50 ml conical tube. Repeat the process once with fresh media with antibiotic-antimycotic mix;
2. After 5 min, transfer the tissue using a 25 ml pipette to another 50 ml tube; this will start the mechanical trituration of the tissue. Add around 25 ml of Accutase 1×;
3. Triturate the tissue using a 10 ml pipette with ten times up and down movements in the same 50 ml with Accutase. Leave the 50 ml tube in the incubator (37 °C) for 10 min;
4. Repeat the trituration process using a 10 ml pipette ten more times;
5. Set a P1000 to 800 μl and with sterile tips, pipette up and down the tissue. If the sample clog the pipette, repeat Step 4. Try to use P1000 to pipette up and down

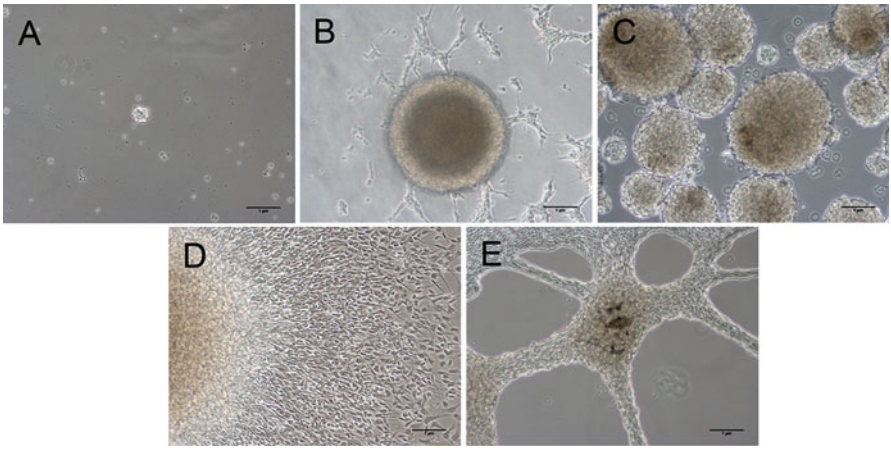

Fig. 4.1 Human neurospheres at different stages in culture. Neurospheres derived from rodent and humans look very similar. The differences occur at distinct time points in the culture, the main one is the doubling time and how long the cells can be maintained *in vitro*. For example: human fetal samples can last more than 2½ years with passages every 2–3 weeks. (**a**) After the dissociation process of the tissue sample, the cells need 1–2 days to reprogram and start to proliferate in both rodent and human species. (**b**) After 14 days *in vitro*, proliferating spheres form a big bright golden sphere. (**c**) Human neurospheres at 21 days *in vitro* are ready to be split. At this point, a combination of small, medium and large spheres can be observed. From day 0 (or the initial isolation for human samples), this step takes 16–21 days. Embryonic samples from rodent take only 2 weeks for initial passage P0 to P1. (**e**) Upon differentiation, neurospheres attach to the precoated surface with Matrigel and start to migrate out from the center in radial patter, and differentiate from the sphere; a similar migration pattern also occurs in rodent. (**e**) After 3 days *in vitro*, the neurospheres attach and, supported by growth and neurotrophic factors, form a network of proliferating spheres with extensions of cells that connect some spheres with others. Scale bar = 1 μm

approximately 20 times, then leave the sample at 37 °C for 5 min, pipette again 30 more times; filter the tissue and cells using a 40 μm filter.

6. Dilute the volume in 15 ml of basal media, sediment at 1200 rpm ($277 \times g$), decant and plate ~2,000–10,000 cells/cm^2 in 2 ml of NPM with FGF/EGF/LIF/B-27 in a 6-well plate. *We strongly recommend the 6-well plate, because paracrine factors help cells to reprogram and proliferate more efficiently if they are in close proximity*;
7. Feed the cells at day 3 and at day 5 or 6, adding FGF, EGF, B-27 and antibiotic-antimycotic. At this point, you should see small proliferation aggregates (Fig. 4.1a). Change half media (50 %) for the first time at day 8 or 9 (Fig. 4.1b);
8. After neurospheres become larger, regular feeding will be once a week; change half media, 2–3 days later, exclusively add FGF/EGF/LIF/B-27 and antibiotic-antimycotic. This feeding schedule applies to the rest of the neurosphere culture;
9. Initial generation and enrichment of the "mature" neurospheres at P0 will take at least 14 to 21 days *in vitro*; after this time, dissociated cells for the next passage.

Subsequent generations of passages are done every 2–3 weeks depending on the growing conditions (Fig. 4.1c).

Chemical and mechanical dissociation of Neurospheres

10. After the neurospheres float in neural proliferation medium for 14–21 days (Fig. 4.1c), transfer them to a sterile 50 ml tube (up to four 6-well plate per 50 ml tube) and centrifuge at 277 × *g* for 7 min;

    Note: *Collect supernatant in a sterile tube as this will be used for the next plating after dissociating neurospheres*
11. Add 1 ml Accutase 1× at room temperature to the cells;
12. Set a P1000 automatic pipetter to 800 µl and with sterile tips, gently pipette up and down the neurospheres for approximately 30 times. Incubate the sample at 37 °C for 10 min (CO_2 incubator), and pipette 30 more times. At this moment, the solution of Accutase and cells will look milk-like;
13. To wash the cells, add 15 ml basal media (no growth factors), sediment at 277 × *g*, decant and split at 1:2 ratio by using another 50 ml tube. Add 50 ml of fresh basal media to the cells, mix, transfer 25 ml with half of the cells in another 50 ml tube. Add 25 ml of used media to each 50 ml tube; you will use half of the cells plus half fresh media and conditioned media. Plate into a 6-well plate. Add FGF/EGF/LIF/B-27 (2,000–10,000 cells/cm^2 in 2 ml NPM);

Note: *Neurosphere are mostly aggregated. We usually do not count cells after passaging and just make a 1:2 split ratio in terms of cm^2 for every 6-well plate or flask that is full of neurospheres, diluting cells and media in two, so that they will grow better in high densities than in higher split ratios.*

14. Once a week, change half media and add FGF/EGF/LIF/B-27 and antibiotic-antimycotic and, 2–3 days later add FGF/EGF/LIF/B-27 and antibiotic-antimycotic (without changing the media);
15. Repeat the dissociation every 2–3 weeks, depending on the number of neurospheres (Fig. 4.1c). As can be seen in Fig. 4.1d, e, the cells migrate and differentiate perpendicular to the center of the neurosphere. The differentiated neurons in the periphery can be analyzed by patch clamp studies to identify variations in the ionic currents under different experimental conditions.

4.6 Potential Use of Human NSCs/NPCs

One of the most compelling arguments for isolation and characterization of human NSCs is its importance in biomedical knowledge and the enormous potential for translational studies in neurodegenerative diseases, including possible cell replacement therapy with an autologous cell source. Thee sources are available to obtain tissue samples for biomedical research and translational studies: (1) fetal samples, (2) post-mortem biopsies and (3) surgical biopsies from elective operations of olfactory epithelia, curative neuro-surgical interventions such as in epilepsy.

Postmortem samples have long been the prime source of human NSCs; some research scientists often argue that the resource is underutilized. Advances in stem cell culture procedures allow postmortem samples to grow stem/progenitor cells in a greater number and for longer periods of time than before. The use of postmortem brain for complex neuropsychiatric diseases is a good example of underutilized biomedical resource (McCullumsmith et al. 2014). Cells from the human olfactory mucosa are another tissue that can be harvested from living human subjects. They regenerate throughout life from neural stem cells. A simple biopsy performed by an otorhinolaryngologist can produce 10–25 cm^2 of epithelial mucosa. This olfactory epithelium can be used for cultures that can be expanded into the large quantities required for molecular and functional analyses, compared to patient-derived *in vitro* model systems of complex neural disease (Matigian et al. 2010).

Acknowledgments This work was supported by NIH (U54NS083924) for AHM and MNG. JLRL received support from the NIGMS (8P20GM103475-12) and MBRS-RISE at UNE (2R25GM066250-05A1) from NIH.

References

Anacker C, Zunszain PA, Cattaneo A, Carvalho LA, Garabedian MJ, Thuret S, Pariante CM (2011) Antidepressants increase human hippocampal neurogenesis by activating the glucocorticoid receptor. Mol Psychiatry 16:738–750

Andres RH, Horie N, Slikker W, Keren-Gill H, Zhan K, Sun G, Manley NC, Pereira MP, Sheikh LA, McMillan EL (2011) Human neural stem cells enhance structural plasticity and axonal transport in the ischaemic brain. Brain 134:1777–1789

Arai Y, Funatsu N, Numayama-Tsuruta K, Nomura T, Nakamura S, Osumi N (2005) Role of Fabp7, a downstream gene of Pax6, in the maintenance of neuroepithelial cells during early embryonic development of the rat cortex. J Neurosci 25:9752–9761

Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O (2002) Neuronal replacement from endogenous precursors in the adult brain after stroke. Nat Med 8:963–970

Barkho BZ, Zhao X (2011) Adult neural stem cells: response to stroke injury and potential for therapeutic applications. Curr Stem Cell Res Ther 6:327–338

Barone FC, Feuerstein GZ, Spera RP (1997) Calcium channel blockers in cerebral ischaemia. Expert Opin Investig Drugs 6:501–519

Brahmachari S, Fung YK, Pahan K (2006) Induction of glial fibrillary acidic protein expression in astrocytes by nitric oxide. J Neurosci 26:4930–4939

Brandt MD, Jessberger S, Steiner B, Kronenberg G, Reuter K, Bick-Sander A, Von Der Behens W, Kempermann G (2003) Transient calretinin expression defines early postmitotic step of neuronal differentiation in adult hippocampal neurogenesis of mice. Mol Cell Neurosci 24:603–613

Braun H, Schäfer K, Höllt V (2002) Beta III tubulin-expressing neurons reveal enhanced neurogenesis in hippocampal and cortical structures after a contusion trauma in rats. J Neurotrauma 19:975–983

Dhara SK, Hasneen K, Machacek DW, Boyd NL, Rao RR, Stice SL (2008) Human neural progenitor cells derived from embryonic stem cells in feeder-free cultures. Differentiation 76:454–464

Dirnagl U, Iadecola C, Moskowitz MA (1999) Pathobiology of ischaemic stroke: an integrated view. Trend Neurosci 22:391–397

Eckenhoff MF, Rakic P (1988) Nature and fate of proliferative cells in the hippocampal dentate gyrus during the life span of the rhesus monkey. J Neurosci 8:2729–2747

Einstein O, Ben-Hur T (2008) The changing face of neural stem cell therapy in neurologic diseases. Arch Neurol 65:452–456

Ellis P, Fagan BM, Magness ST, Hutton S, Taranova O, Hayashi S, McMahon A, Rao M, Pevny L (2004) SOX2, a persistent marker for multipotential neural stem cells derived from embryonic stem cells, the embryo or the adult. Dev Neurosci 26:148–165

Endo M, Doi R, Nishita M, Minami Y (2012) Ror family receptor tyrosine kinases regulate the maintenance of neural progenitor cells in the developing neocortex. J Cell Sci 125:2017–2029

Eriksson PS, Perfilieva E, Björk-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH (1998) Neurogenesis in the adult human hippocampus. Nat Med 4:1313–1317

Gingras M, Champigny MF, Berthod F (2007) Differentiation of human adult skin-derived neuronal precursors into mature neurons. J Cell Physiol 210:498–506

Graham V, Khudyakov J, Ellis P, Pevny L (2003) SOX2 functions to maintain neural progenitor identity. Neuron 39:749–765

Hall PE, Lathia JD, Miller NG, Caldwell MA, Ffrench-Constant C (2006) Integrins are markers of human neural stem cells. Stem Cells 24:2078–2084

Hansen DV, Lui JH, Parker PRL, Kriegstein AR (2010) Neurogenic radial glia in the outer subventricular zone of human neocortex. Nature 464:554–561

Huang J, Upadhyay UM, Tamargo RJ (2006) Inflammation in stroke and focal cerebral ischemia. Surg Neurol 66:232–245

Iadecola C, Alexander M (2001) Cerebral ischemia and inflammation. Curr Opin Neurol 14:89–94

Itoh T, Satou T, Ishida H, Nishida S, Tsubaki M, Hashimoto S, Ito H (2009) The relationship between SDF-1alpha/CXCR4 and neural stem cells appearing in damaged area after traumatic brain injury in rats. Neurol Res 31:90–102

Kim M, Habiba A, Doherty JM, Mills JC, Mercer RW, Huettner JE (2009) Regulation of mouse embryonic stem cell neural differentiation by retinoic acid. Dev Biol 328:456–471

Kirik OV, Vlasov TD, Korzhevskii DE (2013) Neural stem cell markers nestin and musashi-1 in rat telencephalon cells after transient focal ischemia. Neurosci Behav Physiol 43:587–591

Liu Y, Namba T, Liu J, Suzuki R, Shioda S, Seki T (2010) Glial fibrillary acidic protein-expressing neural progenitors give rise to immature neurons via early intermediate progenitors expressing both glial fibrillary acidic protein and neuronal markers in the adult hippocampus. Neurosci.166:241–251

Lu QR, Yuk D, Alberta JA, Zhu Z, Pawlitzky I, Chan J, McMahon AP, Stiles CD, Rowitch DH (2000) Sonic hedgehog—regulated oligodendrocyte lineage genes encoding bHLH proteins in the mammalian central nervous system. Neuron 25:317–329

Marmur R, Mabie PC, Gokhan S, Song Q, Kessler JA, Mehler MF (1998) Isolation and developmental characterization of cerebral cortical multipotent progenitors. Dev Biol 204:577–591

Martins AH, Alves JM, Trujillo CA, Schwindt TT, Barnabe GF, Motta FLT, Guimaraes AO, Casarini DE, Mello LE, Pesquero JB, Ulrich H (2008) Kinin-B2 receptor expression and activity during differentiation of embryonic rat neurospheres. Cytometry 73:361–368

Martins AH, Hu J, Xu Z, Mu C, Alvarez P, Ford BD, El Sayed K, Eterovic VA, Ferchmin PA, Hao J (2015) Neuroprotective activity of (1S,2E,4R,6R,-7E,11E)-2,7,11-cembratriene-4,6-diol (4R) in vitro and in vivo in rodent models of brain ischemia. Neuroscience 291:250–259

Matigian N, Abrahamsen G, Sutharsan R, Cook AL, Vitale AM, Nouwens A, Bellette B, An J, Anderson M, Beckhouse AG, Bennebroek M, Cecil R, Chalk AM, Cochane J, Fan Y, Feron F, McCurdy R, McGrawth JJ, Murrell W, Perry C, Raju J, Ravishankar S, Silburn PA, Sutherland GT, Mahler S, Mellick GD, Wood SA, Sue CM, Wells CA, Mackay-Sim A (2010) Disease-specific, neurosphere-derived cells as models for brain disorders. Dis Model Mech 3:785–798

McCullumsmith RE, Hammond JH, Shan D, Meador-Woodruff JH (2014) Postmortem brain: an underutilized substrate for studying severe mental illness. Neuropsychopharmacology 39:65–87

Messam CA, Hou J, Major EO (2000) Coexpression of nestin in neural and glial cells in the developing human CNS defined by a human-specific anti-nestin antibody. Exp Neurol 161:585–596

Miralles VJ, Martínez-López I, Zaragozá R, Borrás E, García C, Pallardó FV, Viña JR (2001) Na+ dependent glutamate transporters (EAAT1, EAAT2, and EAAT3) in primary astrocyte cultures: effect of oxidative stress. Brain Res 922:21–29

Mitome M, Low HP, van den Pol A, Nunnari JJ, Wolf MK, Billings-Gagliardi S, Schwartz WJ (2001) Towards the reconstruction of central nervous system white matter using neural precursor cells. Brain 124:2147–2161

Nagato M, Heike T, Kato T, Yamanaka Y, Yoshimoto M, Shimazaki T, Okano H, Nakahata T (2005) Prospective characterization of neural stem cells by flow cytometry analysis using a combination of surface markers. J Neurosci Res 80:456–466

Nait-Oumesmar B, Picard-Riera N, Kerninon C, Decker L, Seilhean D, Hoglinger GU, Hirsch EC, Reynolds R, Baron-Van Evercooren A (2007) Activation of the subventricular zone in multiple sclerosis: evidence for early glial progenitors. Proc Natl Acad Sci U S A 104:4694–4699

Neuman T, Keen A, Zuber MX, Kristjansson GI, Gruss P, Nornes HO (1993) Neuronal expression of regulatory helix-loop-helix factor Id2 gene in mouse. Dev Biol 160:186–195

Ohnishi A, Sawa H, Tsuda M, Sawamura Y, Itoh T, Iwasaki Y, Nagashima K (2003) Expression of the oligodendroglial lineage-associated markers Olig1 and Olig2 in different types of human gliomas. J Neuropathol Exp Neurol 62:1052–1059

Park D, Xiang AP, Mao FF, Zhang L, Di CG, Liu XM, Shao Y, Ma BF, Lee JH, Ha KS (2010) Nestin is required for the proper self-renewal of neural stem cells. Stem Cells 28:2162–2171

Park HJ, Bronson RT, Frankel WN, Hong M, Israel MA, Yun K (2013) Elevated Id2 expression results in precocious neural stem cell depletion and abnormal brain development. Stem Cells 31:1010–1021

Pfenninger CV, Roschupkina T, Hertwig F, Kottwitz D, Englund E, Bengzon J, Jacobsen SE, Nuber UA (2007) CD133 is not present on neurogenic astrocytes in the adult subventricular zone, but on embryonic neural stem cells, ependymal cells, and glioblastoma cells. Cancer Res 67:5727–5736

Raponi E, Agenes F, Delphin C, Assard N, Baudier J, Legraverend C, Deloulme JC (2007) S100B expression defines a state in which GFAP-expressing cells lose their neural stem cell potential and acquire a more mature developmental stage. Glia 55:165–177

Rodriguez-Martinez G, Molina-Hernandez A, Velasco I (2012) Activin A promotes neuronal differentiation of cerebrocortical neural progenitor cells. PloS One 7:e43797

Seki T (2002) Expression patterns of immature neuronal markers PSA-NCAM, CRMP-4 and NeuroD in the hippocampus of young adult and aged rodents. J Neurosci Res 70:327–334

Son MJ, Woolard K, Nam DH, Lee J, Fine HA (2009) SSEA-1 is an enrichment marker for tumor-initiating cells in human glioblastoma. Cell Stem Cell 4:440–452

Sun Y, Kong W, Falk A, Hu J, Zhou L, Pollard S, Smith A (2009) CD133 (Prominin) negative human neural stem cells are clonogenic and tripotent. PloS One 4:e5498

Suzuki S, Namiki J, Shibata S, Mastuzaki Y, Okano H (2010) The neural stem/progenitor cell marker nestin is expressed in proliferative endothelial cells, but not in mature vasculature. J Histochem Cytochem 58:721–730

Terkelsen OB, Stagaard Janas M, Bock E, Møllgård K (1992) NCAM as a differentiation marker of postmigratory immature neurons in the developing human nervous system. Int J Dev Neurosci 10:505–516

Tohyama T, Lee VM, Rorke LB, Marvin M, McKay RD, Trojanowski JQ (1992) Nestin expression in embryonic human neuroepithelium and in human neuroepithelial tumor cells. Lab Invest 66:303–313

Trujillo CA, Schwindt TT, Martins AH, Alves JM, Mello LE, Ulrich H (2009) Novel perspectives of neural stem cell differentiation: from neurotransmitters to therapeutics. Cytometry A 75:38–53

Uchida N, Buck DW, He D, Reitsma MJ, Masek M, Phan TV, Weissman IL (2000) Direct isolation of human central nervous system stem cells. Proc Natl Acad Sci 97:14720–14726

Venere M, Han YG, Bell R, Song JS, Alvarez-Buylla A, Blelloch R (2012) Sox1 marks an activated neural stem/progenitor cell in the hippocampus. Development 139:3938–3949

Vescovi AL, Parati EA, Gritti A, Poulin P, Ferrario M, Wanke E, Galli R (1999) Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. Exp Neurol 156:71–83

Xu Z, Jiang J, Ford G, Ford BD (2004) Neuregulin-1 is neuroprotective and attenuates inflammatory responses induced by ischemic stroke. Biochem Biophys Res Commun 322:440–446

Young MJ, Ray J, Whiteley SJO, Klassen H, Gage FH (2000) Neuronal differentiation and morphological integration of hippocampal progenitor cells transplanted to the retina of immature and mature dystrophic rats. Mol Cell Neurosci 16:197–205

Yun SW, Leong C, Zhai D, Tan YL, Lim L, Bi X, Lee JJ, Kim HJ, Kang NY, Ng SH, Stanton LW, Chang YT (2012) Neural stem cell specific fluorescent chemical probe binding to FABP7. Proc Natl Acad Sci 109:10214–10217

Zhang RL, Zhang ZG, Zhang L, Chopp M (2001) Proliferation and differentiation of progenitor cells in the cortex and the subventricular zone in the adult rat after focal cerebral ischemia. Neuroscience 105:33–41