Efficient Generation of Viral and Integration Free Human Induced Pluripotent Stem Cell-Derived Oligodendrocytes

Araceli Espinosa-Jeffrey[1], **Bruno Blanchi**[1], **Juan Carlos Biancotti**[2], **Shalini Kumar**[1], **Megumi Hirose**[1], **Berhan Mandefro**[3], **Dodanim Talavera**[3], **Nissim Benvenisty**[4], and **Jean de Vellis**[1]

[1]Intellectual and Developmental Disabilities Research Center, Semel Institute for Neuroscience, David Geffen School of Medicine at UCLA, Los Angeles, CA

[2]Zilkha Neurogenetic Institute, Keck School of Medicine, University of Southern California, Los Angeles, CA

[3]Regenerative Medicine Institute, Cedars Sinai Medical Center, Los Angeles, CA

[4]Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel

Abstract

Here we document three highly reproducible protocols: 1) A culture system for the derivation of human oigodendrocites (OLs) from human induced pluripotent stem cells (hiPS) and their further maturation; Key features are the concomitant fate restriction, and lineage specification of hiPS towards the neutral and OL phenotypes. The use of very precise amounts of fresh additives and factors also contributes to the shorter periods of time (56days vs. /200days/75days) necessary to obtain hiPS-derived OLs. Our protocol generates viral and integration free OLs that efficiently commit and move forward in the OL lineage recapitulating all the steps known to occur during *in vivo* development. 2) Protocols for the isolation, propagation and maintenance of neural stem cells (NSCs); and 3) Protocols for the production, isolation, and maintenance of OLs from perinatal rodent and human brain-derived NSCs. Our unique culture systems rely on a series of chemically defined media, specifically designed and carefully characterized for each developmental stage of OL as they advance from OL progenitors to mature, myelinating cells. We are confident that these protocols bring our field a step closer to efficient autologous cell replacement therapies and disease modeling.
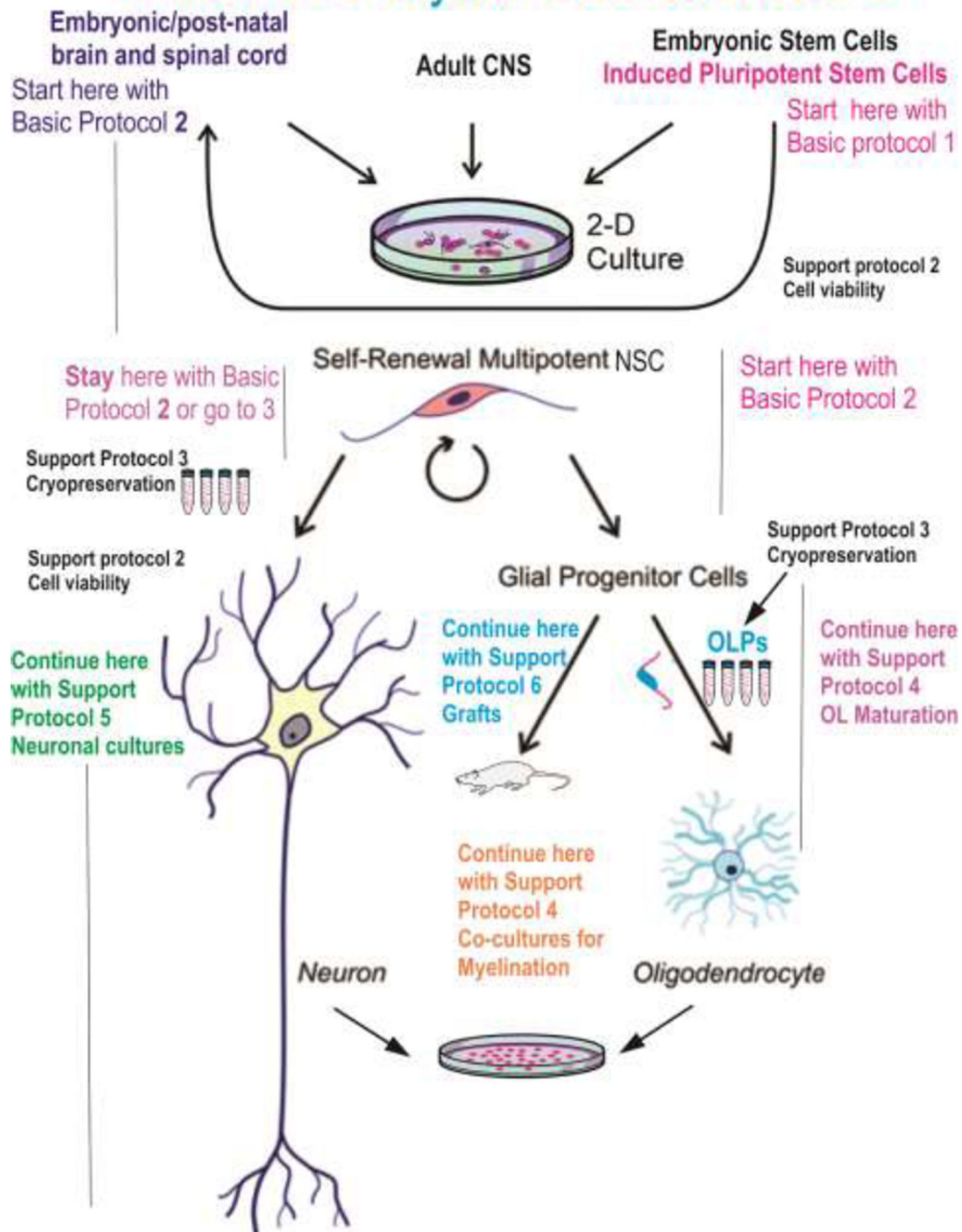
Graphical Abstract

*Corresponding Author: Araceli Espinosa-Jeffrey, Ph. D., University of California, Los Angeles, Neurosciences Research Building, Room 260G, 635 Charles E. Young Drive South, Los Angeles, CA 90095-7332, Tel: 310-825-1747; Fax: 310-206-5061, aespinosa@mednet.ucla.edu.

Which one is your source of cells?

Embryonic/post-natal brain and spinal cord

Start here with Basic Protocol 2

Adult CNS

Embryonic Stem Cells
Induced Pluripotent Stem Cells

Start here with Basic protocol 1

2-D Culture

Support protocol 2 Cell viability

Self-Renewal Multipotent NSC

Stay here with Basic Protocol **2** or go to 3

Start here with **Basic Protocol 2**

Support Protocol 3 Cryopreservation

Support Protocol 3 Cryopreservation

Support protocol 2 Cell viability

Glial Progenitor Cells

Continue here with Support Protocol 6 Grafts

OLPs

Continue here with Support Protocol 4 OL Maturation

Continue here with Support Protocol 5 Neuronal cultures

Continue here with Support Protocol 4 Co-cultures for Myelination

Neuron

Oligodendrocyte

This figure indicates the different sources of neural cells depending of the starting material i.e. embryonic/neonate or adult brain, ES cells, or induced pluripotent stem cells. The starting material would determine where on this chart the user would start the cultures to make oligodendrocytes.

Keywords

Human induced pluripotent stem cells; neural stem cells; NSC; oligodendrocyte specification; oligodendrocyte maturation; lineage progression; chemically defined media; oligospheres; neurospheres

INTRODUCTION

In embryonic life, pluripotent cells proliferate and commit into distinctive cell lineages. During development, *in vivo* lineage commitment occurs and is maintained by the epigenetic programing of gene expression profiles in which methylation plays a prominent role (Goll and Bestor, 2005). In cell culture, somatic differentiated cells can be made pluripotent with the use of specific pluripotency genes like Oct4 and Nanog. (Takahashi and Yamanaka, 2006). During commitment/differentiation, these genes undergo silencing by de novo DNA methylation in their promoter and enhancer regions maintaining thereafter their hyper-methylated state as differentiated somatic cells (Li et al., 2007). Differences have been found in commitment/differentiation potentials among human pluripotent cell lines and therefore, the culture media can be adjusted, depending on the particular cell line/type being used, to provide the desired results. Our goal was to obtain a "culture system" to implement non-genetic yet stable and irreversible cell commitment. The defined culture medium should contain instructive and selective molecules. There is a lot of interest in deriving OL progenitors (OLPs) from hiPS for cell replacement therapies (Goldman S., 2011) in a shorter period of time than 200 days (Sim et al., 2009). Several protocols have been published aiming at the same goal and they include the use of growth factors and small molecules. More recently it has been reported that OLs can be generated from fibroblasts donated by multiple sclerosis (MS) patients (Douvaras et al., 2014), we appreciate the literature yet it would be inappropriate to include a review of all the literature available in this protocol. We have based our method on three main publications as well as, on the experience we have developed in our laboratory (that expands well over four decades) on the needs of oligodendrocytes as they commit and develop to become functional myelinating cells.

The first publication (Kim et al., 2010) describes a robust enhancement of neural differentiation from human ES and iPS regardless of their innate difference in commitment propensity. The authors used the small molecules ROCK inhibitor, dorsomorphin, and SB431542. In the protocol described herein we shortened their use. Mo and Zecevic (2009) had shown that the numbers of O4-expressing OL progenitors increase when using sonic hedgehog (Shh) in their cultures. Several other authors have used Shh and also retinoic acid (RA) in their medium and in particular, Hu et al., (2009) in their paper described that human OLs derived from ES conserve Shh signaling networks with divergent basic fibroblast growth factor (bFGF) effects. Thus, we incorporated the use of both Shh and RA. The main advantage over any other media described in the literature to generate OLs from human ES or iPS is that with the medium described here OLPs appear much faster. We previously devised a culture system for the production, isolation and maintenance of the OL phenotype from rodent and human neural stem cells (NSC; Espinosa et al., 2009). Here we expand the information and document a protocol for the specification of hiPS to the OL phenotype

based on the information we have previously published. Our unique method is reliable because it uses our previously described *series of chemically defined media*, specifically designed, and carefully *characterized for each developmental stage* of OLs, as they advance from OLPs to mature myelinating OLs [Figure 1] (Neman and de Vellis, 2008; Espinosa et al., 2009).

Since our previous chapter describes our "cell culture system" that instructs NSCs to become OLs departing from perinatal brain, in the current chapter we describe the specific nutrients, additives and time windows when the additives should be provided to instruct hiPS to become OLs. We start at the moment when "embryoid bodies (EBs)" are prepared and thereafter to direct and instruct their commitment to the OL phenotype. Once commitment of hiPS cells to OLs has been achieved, the steps of lineage progression is well supported by the culture system we already described (Espinosa et al., 2009). *Propagation of hiPS-derived OLs at a desired stage* from OLPs to mature premyelinating OLs as well as, lineage progression can be manipulated by controlling the duration of a given developmental stage as needed, in a more "natural" manner, and without using additional gene transfer (Park et al., 2002b; Müller et al., 2006; Ahn et al., 2008), co-cultures, or undefined substrates such as "a different cell line-derived conditioned medium (CM)" or animal serum.

BASIC PROTOCOL 1

> I.   **Preparation of EBs from hips while starting neural instruction**
>
> II.   **Preparation of EBs from hiPS while starting neural instruction**

Step 1 EBs Preparation

Materials: (See Table 1)

> Mouse embryonic fibroblast (MEF) medium
>
> EB medium (Note 1)
>
> hiPS medium (Note 2)
>
> N2 basal medium
>
> Trypsin/EDTA 0.25%
>
> DPBS
>
> 60 mm low cell binding culture dishes (NUNC, catalog number 145389)
>
> 5mM ROCK inhibitor (1000× stock)
>
> 10mM Dorsomorphin (1000× stock)
>
> Matrigel 1/20 (Note 3)
>
> Incubator set to 5% CO_2

Procedures: (Figure 1)

1.   Culture hiPS on MEFs in six-well plates following standard protocol (Biancotti and Lavon, 2012), until they reach 80–90% confluence.

2.   Prior to preparing the EBs, pre-treat the hiPS cultures with ROCK inhibitor (1μl/ml) in ES medium for 2 h to 6 h.

3.   Remove the old medium from the cells and rinse each well with DPBS.

4.   Aspirate the DPBS and add 0.5 ml of freshly prepared warm trypsin-EDTA (Note 4) to each well of the six-well plate.

5.   Incubate the cultures at room temperature for 1–2 min, until cells start detaching.

6.   Add 0.5 ml of MEF media to each well to inactivate the trypsin, and collect the cells into 15 ml conical tubes, pooling up to 4 wells per tube. Spin them down for 5 min at 400 xg.

7.   Resuspend the cells in 5 ml of EB medium and spin it down to wash the remnant trypsin.

8.   Repeat step 7 one more time.

9.   Resuspend the cells in 3 ml of EB media per well pooled in each tube, supplemented with double amount of ROCK inhibitor (2 μl/mL).

10.   Homogenize the cell suspension by gently pipetting up and down, and distribute 3 ml of the suspension per each low-binding culture dish, so to obtain 1 well/dish ratio.

11.   Add 3 ml of EB media supplemented with double amount of dorsomorphin (2 μl/mL) and SB431542 (2 μl/mL) to each dish to complete 6 ml.

12.   Allow EBs to grow for 2 days without changing the media.

13.   Next day, leave EBs undisturbed.

14.   On the second day, remove 3 ml of media by carefully tilting the dish and aspirating from the surface, and replace same volume of fresh EB media containing double amount of dorsomorphin (2 μl/mL) and SB431542 (2 μl/mL), but **not** ROCK inhibitor. Gently tilt forward and backward, and from side to side to mix the media and redistribute the EBs.

15.   Next day, EBs should remain undisturbed (Note 5).

NOTE: KOSR performance differs considerably between lots. It is strongly advised to test different lots in parallel to the lot in use, to select the most supportive KOSR.

NOTE: bFGF is very unstable and rapidly degrades at room temperature. Warm up to room temperature only the aliquot of PS medium to be used, keeping always the rest of the medium at +4°C.

NOTE: To prevent gelification, Matrigel has to be thawed on ice. Use cold DPBS to make 1/20 dilution. Cool down pipettes and tips by aspirating cold DPBS solution prior to use with Matrigel, and culture plates or flasks by keeping them on ice. Once diluted, add Matrigel suspension to the culture dish and incubate at room temperature for 2 h. Following aspiration of suspension, dishes are ready to be used. If not used immediately, sealed them with Parafilm and store them in a plastic bag at 4°C.

NOTE: Trypsin auto-degrades when warm. After thawing, keep solution always cold and warm up to 37°C only the aliquot to be used.

NOTE: If the EBs are too small, just a few cells grouped (< 30–40 μm diameter), you may wait one or two more days before moving to next step.

Step 2. Establishing the Neural and OL phenotypes (Figure 2 and 3)

Materials

> Matrigel or IgM coated T-75 or T-115 coated flask(s)
>
> N2 medium
>
> GDM culture medium
>
> Incubator set at 4.5% CO_2

To be used immediately prior to plating/feeding EB cells

> Shh 250 μg/ml (frozen stock aliquoted)
>
> bFGF 100 μg/ml (frozen stock aliquoted)
>
> RA 100 mM in DMSO
>
> N2 supplement
>
> B-27supplement

Preparing OLBN immediately prior to being used

> GDM + B-27 +N2 = OLBN

Procedures

1. On day 4–5, aspirate media and EBs gently, place them in a 15 ml conical tube and let them sink by gravity for 1–2 min (Note 5).

2. Aspirate the supernatant gently and re-suspend the EBs in 5 ml of N2 medium.

3. Repeat steps 1 and 2 one more time to eliminate death cells and cell debris.

4. Plate EBs on matrigel-coated plates, one 60 mm dish into 1 well of a 6 well plate, a T-75, or a T-115 flask in N2 medium supplemented with 20 ng/ml of bFGF, 250 ng/ml of Shh, and 100 nM of RA, **at this point**

discontinue the use of bFGF. **This is considered day 0,** next day leave cultures undisturbed.

5. **On day 2**, very slowly and without shaking the flask, add OLBN with Shh and RA at a volume ratio of 1:1 (for the entire volume of medium in the flask). **On day 4**, remove 2/3 of the culture medium very slowly and without shaking the flask and replace it with the same volume of GDM.

6. **On day 6 or 7,** remove the culture medium and place it in a tube or clean container. Measure the volume and extremely carefully add an equal volume of fresh OLBN to the flask. At this point (day 6 or 7), you should discontinue the Shh and RA.

7. From now on, you may maintain these cells in OLBN and feed the cultures every 4th day. OLPs will still proliferate; numerous cells will generate from the mother clone and grow separately. Feed by adding OLBN for the next 4 days.

    You may start harvesting clones to be re-plated in cell culture containers for your studies, in which case you may re-plate the clone(s) on poly-D-lysine or Matrigel. Alternatively, you may re-plate the clones on anti-human PSA-CAM or anti- human IgM-coated flasks for their propagation. To continue propagating the cells use OLBN.

8. **After 8 days**, to continue instructing progenitors toward the OL fate, as well as propagating already specified OLPs, you may remove 1/2 of the volume of conditioned medium and add the same volume of OLBN. If the culture is too rich and the medium becomes orange within 3 days, you may add a larger volume of medium to maintain the medium red rather than orange. Cells will continue to give OLP clones in the original flask at least for 2 months. It is recommended that if the cultures are rich in clones, you pass the clones manually without bringing any flat cells, and re-plate them to either work with them or create a frozen stock (Note 6).

Step 3. Choosing and re-plating single clones

Materials

Poly-D-lysine-coated coverslips, flasks, 24 well plates, 4 well plates, or 16 well glass chambers

OLBN (to continue propagating OLPs) or GDM

IGF-1(88 to 100 μg/ml stock), use 1μl/ml of medium

T3 (20 μg/ml stock solution)

GDM culture medium

IGF-1(88 to 100 μg/ml stock)

T3 (20 μg/ml stock solution)

Procedures

1.   Pick up clones that are clear or partially clear as those shown in Figure 3C with a yellow tip, deposit them on the surface of the new container and let them sit for 5 to 10 min (the tip carries some medium with the clones so they won't dry out) (Note 6).

2.   As long as the mother flask is exposed to fresh OLBN, these committed progenitors will continue proliferating and new clones will grow as shown in Figure 4A. These cultures can be re-plated several times (Figures 4C, D and E).

3.   For these OLPs to move forward on the lineage, slowly add the culture medium GDM + IGF-1 (final concentration of 88 –100 ng/ml, freshly prepared) and let the culture sit for 7 to 10 min in the clean bench prior to transfer it to the incubator.

> *In order to continue expanding the OL population at this stage, you will need to supplement the GDM with IGF-1 every third day at the same concentration, until the flask or well is confluent. Do this by removing 1/2 of the culture medium and add the same volume of fresh medium containing the full amount of IGF-1 to replenish the entire volume of culture medium. Alternatively, you may feed GDM + IGF-1 only once, and after 4 days you may add GDM with T3 at a final concentration 40 ng/ml triiodothyronine (Sigma-Aldrich, St. Louis, MO). A chart illustrating the steps and culture media that is instructive for pluripotent cells to neural and OL commitment is shown in Figure 5.*

4.   Clones should not be disrupted neither mechanically nor enzymatically as they cannot be dissociated without damaging many cells. Moreover, they would form rosary-like floating threads and therefore, they will not anchor properly on the substrate, and as a result they will not mature. Instead, fish the clone and seed it on the new cell culture container. The cells will slowly migrate out and still proliferate while being fed with fresh OLBN.

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Support Protocol 1

Immunopanning (alternative to Matrigel)

We developed the following method based on published work (Wysocki and Sato, 1978, and Williams and Gard, 1997). To isolate the rodent and human NSC population from other cell populations of the brain, we used anti-human PSA-NCAM IgM coated dishes during the initial plating. During primary derivation of human NSCs, this method has proven useful

(Wakeman et al., 2009a). Here we propose to use IgM coating for the isolation and derivation of OL from hiPS embryoid bodies as an alternative approach to Matrigel.

Materials

Anti-human PSA-NCAM IgM

Bovine Serum Albumin (BSA)

Flasks (T-12.5 cm^2, T-75 cm^2), or Tissue Culture Flask T-115 (TPP) with recloseable lid

PBS

Procedures

1. Prepare the immunopanning cocktail by mixing: Tris (pH 9.5) + 1% BSA + 50g/ml anti-human PSA-NCAM.

2. Coat the bottom surface of the flask with anti-human PSA-NCAM mixture (4 to 5 ml per dish).

3. Incubate for 30 min at 37°C.

4. Wash flasks 3 times with PBS and once with PBS + 1% BSA just before using. Do not allow the flasks to dry.

5. Extra flasks may be covered with foil and stored at 4°C for up to 10 days.

Support Protocol 2

Cell Viability Assay

Cell viability can be determined with the SYTOX blue nucleic acid stain (Molecular Probes, Eugene, OR). Cells with compromised plasma membranes are labeled by SYTOX binding to nucleic acids and detected by fluorometry.

Materials

PBS

SYTOX blue nucleic acid stain

Tris-Buffered Saline (TBS)

Procedures

1. Harvest and wash cells with 1× TBS.

2. Incubate for 12 min in 1M SYTOX in PBS.

3. Remove the solution and wash the cells with 1× TBS, five times.

4. Determine the *number of positive cells per random field* and record as a percentage of the total number of cells.

Support Protocol 3

We recommend collecting cells for frozen stocks at low passage number. Human NSCs are cryopreserved using modified methods found elsewhere (Wakeman et al., 2009a). In addition, the method formerly described for rat and mouse NSCs (Espinosa et al., 2002) can also be used to stock human NSCs.

A) Freezing NSCs

Materials

Cryogenic slow-freezing chamber

Cryogenic freezing vials

HBSS

STM media

14-gauze needle

Procedures

1. Allow NSCs to grow to 70–90% confluency. Remove all of the cell culture medium, add 5 ml of HBSS to each Petri dish or 10 ml to T-75 flasks, and detach cells by gently scraping the culturing surface.

2. Centrifuge the cells at 45 xg for 8 min and resuspend in 3 ml of STM media.

3. Gently dissociate cells using a 14-gauge needle, pellet at 45 xg for 8 min in a centrifuge, and discard the supernatant.

4. Gently resuspend the pellet from one 100 mm Petri dish or T-75 flask in 1 ml of serum-free freezing medium.

5. Transfer the content to a 1.2 ml cryovial, and place the vial(s) in a cryogenic freezer container overnight for slow freezing.

6. Next day, place the vials in liquid nitrogen for long-term storage.

B) Thawing NSCs

Materials

Anti-human PSA-NCAM, anti-human whole IgM molecule or poly-D-lysine coated cell culture containers

CM and STM media

NSC Cryovial

Procedures

1. To "reanimate" NSCs, defrost cryovials quickly in a 37°C water bath, and transfer the content of the vial to a 2 ml tube containing 1 ml of STMc + CM at 37°C.

2. Centrifuge gently at 35 xg for 5–7 min.

3. Remove the supernatant, add fresh plating medium (STMc + CM) and remove a small aliquot to test the initial cell viability (as described above).

4. Count the number of viable cells in the tube (approx. 1×10^6 cells expected).

5. Plate cells onto anti PSA-NCAM-coated surface (Petri dishes or T-75 cm^2 flasks, plate the equivalent of 1 vial/T-75 cm^2 flask). If the yield is low, utilize T-25 cm^2 flasks to increase the cell density necessary for healthy growth. Seeding low-density cultures in large containers decreases the proliferation rate and might be detrimental to the culture.

6. To propagate NSCs after replating, proceed as described in step 2 for "propagation and maintenance of NSCs".

NOTE: If cells seem not to grow but look healthy, or if the culture medium is not red but rather purple, you will need to remove 1/2 of the plating medium and complete to 10 ml volume of the mixture (1/3 self-conditioned-STM + 2/3 of fresh STMc freshly prepared). If the opposite is true and the culture medium turns orange overnight, the cells have proliferated heavily, and you will need to replace the entire culture medium and seed more T-75 cm2 flasks (per three T-12.5 cm2).

NOTE: When propagating cells to create frozen stocks, we strongly recommend maintaining a "mother flask/dish" by scraping most, but not all of the cells attached to the flask. After removing the detached cells, feed the mother flask with fresh medium and CM (1:1) to ensure continuity of these cultures (in case re-plated cells do not look healthy, grow slowly or die).

BASIC PROTOCOL 2

Propagation of Rodent and Human Neural Stem Cells (NSCs) and derived OLs (Figure 6)

For details on the the isolation of NSCs from rodent and human brain please refer to Espinosa et al., 2009

OL specification in 2D cultures (Figure 7)

During development, the nutritional and environmental needs of cells change as they lose multipotency and become lineage restricted. The present system is based on the modification of nutrients contained in the cell culture medium and the percentage of CO_2 needed to optimize and direct lineage restriction towards the OL phenotype. Like NSCs, OLPs can be propagated in 2D and 3D cultures. When attached (2D cultures), OLPs grow faster, and thus

ideal to create an OLP cell stock quickly before starting specific *in vitro* cell culture or *in vivo* transplantation studies. A diagram of the following steps can be found in Figure 7.

Materials

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Materials

Media: OSM, GDM, OLDEM

Anti-human PSA-NCAM IgM coated plates

bFGF

Erlenmeyer (EM) flask

HBSS without Ca^{2+} or Mg^{2+}

Petri dishes

14-gauge needle

15 μm sieve

24-well plate

4.5% CO_2 incubator

Procedures

1. When NSCs reach confluency, remove the supernatant (CM), and add 5 ml of Hank's Buffered Salt Solution (HBSS) without Ca^{2+} or Mg^{2+}.

2. Detach the cells with a cell scraper, transfer into a 15 ml tube (if you have 1 to 3 Petri dishes), rinse once with 2 ml of HBSS, and centrifuge at 45 xg for 8 min.

3. Resuspend the cell pellet in 3 ml OSM media and gently dissociate (3×) using a 14-gauge needle. Centrifuge at 45 xg to pellet the cells.

4. Resuspend the cells in fresh OSM + STM-CM and seed cells on anti-IgM coated dishes or flasks (use the same procedure as for anti-PSA-NCAM), and maintain the cells as described in *Basic Protocol 1* but using OSM/ self-CM (2:1) respectively. From this point on, the CO_2 concentration in the incubator should remain at 4.5% (Note 9).

5. Feed the cells with 1/3 self-conditioned-OSM + 2/3 fresh OSM every other day until they reach 80–90% confluency. This process can be repeated several times to attain a large number of cells for freezing (if desired).

6. Alternatively, to grow OL spheres to create/enrich a frozen stock of OLP, place the equivalent of 2 mm^2 (pellet size after dissociated and in suspension) in a 25 ml Erlenmeyer flask with 15 ml of OSM + self-CM (2:1). If the pellet is 4 mm^2, use a 50 ml Erlenmeyer flask. Prepare the cell suspension and place in a total volume of 25 ml of OSM + self-CM (2:1). Feed OL-spheres with fresh OSM every other day by adding 3 ml of freshly prepared OSM (no CM). When spheres start to become larger than 2 mm, gently dissociate 1–2 times in the same flask with the 14-gauge needle using a 12 ml syringe (sterile).

7. When the culture medium starts to turn orange, recover and centrifuge the spheres, and split into more Erlenmeyer flasks. These may be used for experiments or cryopreserved as previously described (cryopreservation section).

We recommended pre-calibrating the percentage of CO_2 one day before plating the cells. If the incubator is shared with other people or needed at 5% for NSC propagation and maintenance, we recommend using T-flasks for 2D cultures instead of Petri dishes. Close the cap completely and then open it 1/4 of a turn before placing in the incubator at 5% CO_2. For propagation and maintenance of OL-spheres, the Erlenmeyer flask should also be kept open just enough to ensure O_2 / CO_2 exchange. When using 4.5% CO_2, loosen the caps of the flasks until half-way open.

Support Protocol 4

Oligodendrocyte lineage progression and maturation (Figure 8)

The nutritional needs for a committed cell within the OL lineage differ considerably as they progress and mature to the next developmental stage. These cells need to start synthesizing enzymes and proteins related to myelination, therefore, the energy demand is enormous compared to their earlier stage where migration and proliferation are the basic functions. The culture medium "GDM" (glial defined medium) was first designed to maintain 04+ GC +/−, CNP+/− cells (for details see Pre-OL in [Fig.1]). Later, we realized that GDM also induced the transition of OLP to pre-OL (Espinosa et al., 1997).

Materials

Media: OSM, GDM, OLDEM

bFGF

Petri dishes with cells

Poly-D-lysine coated wells/plates

24-well plate

Procedures

1. In order to obtain "pre-OL" (along the OL lineage), plate OLPs using OSM (as in Basic Protocol 2).

2. Next day, remove 1/2 of the plating medium (OSM) volume and add the same volume of GDM. As in previous steps, they may be propagated as OL-spheres (3D) or as 2D cultures on anti-IgM coated flasks, Petri dishes, or directly on cell culture grade plastic.

3. To obtain more OLP/pre-OL, cells are grown as 2D or 3D cultures in the presence of bFGF (Fig. 4). Cells will remain at the same stage as the parent cells by adding fresh GDM + [20 ng/ml] bFGF. For cell replacement therapies, we suggest using cells at this stage (1–2 days after plating without bFGF) as cells are still highly motile and readily migrate within the host post-natal and/or adult rodent brain and/or spinal cord.

4. To enhance maturation of cells into the next developmental stage, OL are cultured as 2D cultures in GDM for at least 2 days (if plated in GDM without bFGF), or 4 days (if plated in GDM + bFGF) without further bFGF supplementation (Figue 5).

5. After exposure to GDM, cells express myelin enzymes and proteins, and they display multipolar, branched cell processes, but not a myelin-like membrane. In addition, OL maintained in GDM for at least 4 days (without bFGF or any other factors) can be further induced to a fully mature myelinating stage.

6. To fully mature OL, plate as 2D cultures onto poly-D-lysine coated wells/plates or Petri dishes in GDM/OLDEM (OL maturation medium) 1:1 for 1–5 days, followed by 100% OLDEM thereafter (Figure 4).

7. Every 4 days, feed the cells by replacing all of the culture medium with fresh OLDEM (Note 10). The medium should look red, not orange. If it turns orange, add more medium while feeding the cells.

NOTE: These cells will express myelin enzyme levels comparable to those found in pure myelin within 5 days after having been introduced to 100% OLDEM. As they mature, cells will synthesize myelin-like membranes *in vitro* even in the absence of neurons. They can be maintained for various weeks if they are sub-confluent, however, if the culture becomes overcrowded, cells will deteriorate and die.

Preparation for Neurons and OLs Co-Cultures (Figure 9)

To perform myelination studies *in vitro*, it is recommended to start with OLs plated on plastic alone (rather than poly-D-lysine) and maintained in GDM for 2 days.

Materials

Media: GDM and OLDEM,

Cell Scraper

Neuronal Co-culture (See below)

25 μm sieve

24-well plates

Procedures

1. Detach cells with cell scraper and centrifuge at 45 xg for 8 minutes in the same culture medium.

2. Remove the supernatant and resuspend the cells in GDM-CM + fresh OLDEM (1:2).

3. A single cell suspension preparation is necessary for this step. Remove any cell clusters with a 25 μm sieve (as described previously).

4. Count the cells and adjust the cell suspension to approximately (200,000 cells/ml) of OLDEM medium.

5. Remove half the volume of culture medium from the neuronal cultures without disturbing the cells (cortical neurons or dorsal root ganglion cells).

6. Slowly add the OL suspension to one 24-well plate containing the neuronal cultures to complete the original total volume in each well.

7. Follow the co-cultures for at least 10 days. To feed, replace 1/2 CM with fresh OLDEM. If the cultures are not overcrowded, they can be kept for at least 4 weeks.

Support Protocol 5

Preparation of Cortical Neurons

Materials

Instruments, animals and materials are the same as described in Basic Protocol 1.

Animals: Time-pregnant (ED14–16) Sprague-Dawley rats (Charles-River).

Bovine Serum Albumin (BSA)

Conical tubes

Culture tubes with cap, Sterile. Fisher 17×100 mm

Hank's Balanced Salt Solution (HBSS)

Hemacytometer

Isoflurane

Needle

Poly-D-lysine

Scissors

Sterile Surgical Gauze

230 μm and 140 μm sieves

24 or 12 well plates

Procedures

1.   Prepare the work area and sterile tools in a biosafety hood (Diagram 1).

2.   Euthanize the rats by isoflurane inhalation.

3.   Extract the placenta containing embryos and place into STM Basal + 1% BSA.

4.   Remove the cerebellum.

5.   Dissect the brain of each embryo and place into Neurobasal-N complete (see Media Preparation).

6.   Separate the cortex from the brain and remove the meninges with forceps.

7.   Combine the cortical tissue of all the brains without meninges. Mechanically dissociate with the needle by gently aspirating the brain pieces and releasing the suspension slowly against the wall of the tube ten times (try to minimize foaming).

8.   Recover the supernatant with the cells in suspension and transfer to a 15 ml tube.

9. Add 2 to 4 ml of Neurobasal-N medium to the chunks left over in the dissociation tube and dissociate again 5 to 8 times.

10. Filter the suspension of dissociated cells through 230 μm and 140 μm sieves to remove cell clusters.

11. Rinse the sieves sequentially with basal Neurobasal-N + 1% BSA at room temperature and add this medium to the tubes containing the cells.

12. Collect the cells by centrifugation in the culture tubes at 40 xg for 8 min.

13. Discard the supernatant very gently as the pellet is very loose.

14. Resuspend the pellet in 4 ml of fresh Neurobasal-N medium with a 5 ml pipette by gently triturating 2 or 3 times. Complete the volume to 12 ml (or the equivalent of 1 embryo/ml) with 2 parts of fresh medium and 1 part of conditioned medium.

15. Assess *cell viability, count cells using a hemacytometer, and plate onto poly-D-lysine coated well plates (Note 12).

16. Incubate plated cells at 37°C in 4.5% CO_2/95% humidity and monitor with a Combustion Test Kit (Bacharach # 10–500) (most electronic panels do not give an accurate reading).

NOTE: Cortical neurons maintained in Neurobasal-N do not need to be fed as frequently as other cell types. Simply add 500 μl of Neurobasal-Nc every third day. Six days after plating, remove 1/4 of the culture medium and add the same volume of fresh Neurobasal-Nc.

NOTE: Cell viability can be determined with SYTOX as described in Cell Viability Assay in Basic Protocol 1.

Support Protocol 6

Transplantation of OL Progenitors into Neonatal Rats

Neural progenitor cells and their differentiated OL counterparts can be stereotaxically transplanted into the newborn developing rat brain relatively non-invasively as previously described (Snyder et al., 1997; Flax et al., 1998; Espinosa et al., 2002). Similar results can be obtained with variations on the transplant method that are more suitable depending on the needs of the host brain and the type of study (Yandava et al., 1999; Ourednik et al., 2001, 2002; Park et al., 2002a; Teng et al., 2002; Wakeman et al., 2006; Redmond et al., 2007). A selection of detailed protocols for neonatal and adult mouse transplantation are described elsewhere (Espinosa et al., 1992; 1993a,b; Lee, 2008; Wakeman et al., 2009a,b; Yan et al., 2004). Upon implantation in the lateral ventricles, donor cells engraft and migrate from the subventricular zone into the host RMS in much the same manner as host NSC.

Materials: (may vary depending on the grafting method of choice)

Aspirator tube assembly (Sigma, A5177–5EA)

Borosilicate glass tube (Sutter Instrument, B100–75-15)

DPBS

Transillumination light source

Microfuge tube with cell sample

Micropipette puller (Sutter Instrument Co., Model P-87)

Neonatal rat (P0–P5)

Warming pad

Warm Water Glove Balloon

Wet Ice

Procedures

1.   Anesthetize the rat pup until the animal no longer retains locomotion or responds to gentle toe and tail pinch. Carefully monitor the pup and proceed to transplantation.

2.   Insert a calibrated, drawn borosilicate glass micropipette into the aspirator tube assembly and rinse the micropipette by drawing and expelling 70% Et-OH five times followed by sterile DPBS ten times to clean the needle.

3.   Gently flick sample in microcentrifuge tube prior to filling the needle, wipe the tube with 70% Et-OH, and uncap the tube.

4.   Slowly draw 4–5 μl cell suspension into the micropipette.

5.   Loosely secure the head of the anesthetized pup and place directly over the light source to visualize the eyes and bregma.

6.   Carefully insert the glass needle into the head at the midline between eye and bregma and slowly inject 2–5 μl cell suspension at 5×10^4 cells/μl into both lateral ventricles. Slowly remove the needle and check for leakage through needle tract. Repeat step 6 into the contralateral hemisphere.

7.   After the injection, warm the pup by placing on a warm water balloon glove or heating pad to increase the body temperature before returning to the mother.

NOTE: In addition to the lateral ventricles, NSC can also be transplanted into the striatum (SN) (Bjugstad et al., 2005, Redmond et al., 2007, and Bjugstad et al., 2008) and corpus callosum (CC). Upon implantation into the CC of the host, HFB-2050 donor cells recognized by the fluorescent Fast Blue (FB) label migrated along the CC and into the CPu (Figure 10). Pre-committed-OL can also be placed locally within focal sites of injury to decrease the need for extensive migration.

Media Preparation

Mouse Embryonic Fibroblast Medium (MEF medium)

Add to 450 ml of DMEM (high glucose with L-glutamine; Sigma cat # 51441C) 50 ml of fetal bovine serum (FBS) and 2.5 ml of penicillin (stock 10,000 U/ml)/streptomycin (10 mg/ml) (Pen/Strep). The medium can be stored at +4°C for up to 2 weeks.

Embryoid Body Medium (EB medium)

Prepare 400 ml of Knock Out-DMEM (KO-DMEM, Life Technologies, cat # 10828-018) + 70 ml of Knock Out Serum Replacement (KOSR, Life Technologies, cat # 10828-028) + 5 ml of 200 mM glutamine + 5 ml of 100× Non-Essential Aminoacids (NEAA) + 2 ml of Insulin-Transferrin-Selenite (ITS) + 40 µl of 1M β-mercaptoethanol (βME) + 2.5 ml of penicillin (stock 10,000 U/ml)/streptomycin (10 mg/ml) (Pen/Strep).

Store EB media at +4°C for up to 2 weeks.

Pluripotent Stem Cell Medium (PS medium)

Add 1.2 ml of 2µg/ml stock of bFGF to 480 ml of EB medium (5 ng bFGF/ml).

Store PS medium at +4°C for up to 1 week.

N2 Basal Medium (N2 medium)

Add to 500 ml of DMEM/F12, 5 ml of N2 supplement (stock 100×), 5 ml of 200 mM L-glutamine, and 5 ml of Pen/Strep.

Store N2 media at +4°C for up to 2 weeks.

Basal Stem Cell Medium (*STM-II; for rat and human NSC)

One liter of D-MEM, SKU # 11054-020 (low glucose, without glutamine, without sodium pyruvate) + 1 liter of Neurobasal medium without normocin, heparin, Vit A or LIF + the following additives:

Insulin	5 mg
Transferrin	50mg
Putrescine	16.1mg
Progesterone	20nM
Sodium Selenium	4µg 0.4mg/ml (4ml/litter of medium)
Sodium Bicarbonate	2.2g
Penicillin-Streptomycin	1 ml
Kanamycin	1 ml

Complete STM (STMc) medium for plating, maintenance and propagation of NSCs

Just before feeding the cells, prepare the desired volume of STM complete by mixing Basal STM and the following supplements to reach the final concentrations as indicated: B-27 (1:50), recombinant Human EGF [20 ng/ml], recombinant human bFGF [20 ng/ml], and Creatine [3.8mg/L].

NOTE: STM-II is a variation of the original STM medium we previously described (Espinosa et al., 2002); UCLA case # 2002-475, which formula is available upon MTA. STM-II yields results comparable to those obtained with STM.

"OSM-II" OL specification medium

Formerly named OTM; (Espinosa et al., 2002); OSM-II derives from STM-II. Add STM-II complete freshly prepared to GDM 1:1 v/v.

"GDM"(Glia defined medium) (Espinosa et al., 1988; 1997)

*One liter of double distilled water + one package D-MEM/F12 medium (High glucose) + the following additives:

Insulin	5 mg	
Transferrin	50 mg	
Putrescine	16.1 mg	
Sodium Bicarbonate	2.2 g	
D(+) galactose	4.6 g	
**Sodium Selenite	8µg Kanamycin	1 ml
*Adjust the pH to 7.4 (after filtering)		
** Prepare a "stock solution" 0.8mg/ml in PBS. Add 8 µl of the stock solution for 1 liter.		

Neurobasal-B27 "NB-B27" (Human Neural Stem Cell Proliferation Medium)

Neurobasal medium	97%
B-27 w/o Vitamin A	2%
GlutaMAX	1%
Normocin (optional)	0.2%
Heparin	[8 µg/mL]
bFGF	[20 ng/mL]
LIF	[10 ng/mL]

"Neurobasal-N" medium for cortical neurons

Neurobasal medium	1 L
Insulin	5 mg/L
Transferrin	50mg/L

Progesterone	20nM *
Sodium Selenite	8μl **
Sodium Bicarbonate	2.2g
Kanamycin	1 ml

---*These additives should be added just before using Neurobasal-N to make complete "Neurobasal-Nc"*

B-27 with vit. A	1:50
Recombinant Human bFGF	[20ng/ml]
Creatine	4mg/ml

COMMENTARY

Background

The culturing system allows for the production of relatively homogeneous primary OL cultures in adequate numbers for cryopreservation. These cell stocks can be used for basic research in further *in vitro* studies. Moreover, these cells are never exposed to animal or human sera, and therefore remain as suitable candidates for cell replacement therapies in developmental disorders of the central nervous system (CNS) as well as neurodegenerative diseases.

Numerous methods and culture media described in the literature (even before, the times of NSC), were the basis for the optimization of the culture media formulations described here (some examples are, Botenstein and Sato, 1979; Saneto and de Vellis, 1985; Espinosa et al., 1988; Yang et al., 2005, Larsen et al., 2008. etc). Undoubtedly, all previous reports on how to obtain and culture OL derived from NSC have also been instrumental in designing the present protocol. For example, the group of Lachapelle, and Baron-Van Evercooren described floating oligospheres derived from newborn rat brain (1996). This concept has been applied to NSC to generate OLs by Zhang et al, 1998, Espinosa et al 2002, and in the present protocol. Zhang and coworkers (1998) described the use of B104 neuroblastoma cell-conditioned medium (B104CM) to induce the oligodendrocyte phenotype on neurospheres and induce proliferation. This approach provides OL for many kinds of studies, but they are unsuitable as donor cells for cell replacement therapies to be used in translational studies, having used uncharacterized conditioned medium from B104 cells that have been grown in the presence of fetal bovine serum (as originally described by Louis et al., 1992). An example on the use of the protocols described can be found in Chattopadhyay et al., 2008.

Critical Parameters

We want to emphasize that fate restriction towards commitment from NSC to OLP (as defined in *"Basic Protocol 2"*) becomes *irreversible* after NSCs have been in OSM for at least 20 hours (2D and 3D cultures). Therefore, the progeny of these cells will define a

homogeneous OLP population, ideal for biochemical, toxicological and pharmacological studies, as well as an appropriate and reproducible source of committed cells to be used in cell therapy studies. Phenotype reversal of induced OLPs may be possible with genetic manipulation but we have not attempted such studies to date.

HuNSC-derived OLs and hiPS-derived OLs are more sensitive to CO2 variations than rodent and primate non-human OLs. Thus monitoring of your incubator with Fyrite is essential for the yield of homogeneous populations of OLs and disfavor of astrocytes and fibroblasts.

Always monitor the concentration of CO_2 with a Combustion Test Kit (Bacharach # 10– 500), as most electronic panels do not provide an accurate reading. The proper lineage progression relies on precise control of CO_2 to maintain a pH that should remain accurate and controlled.

Troubleshooting

Human NSC are more fragile than their rodent counterparts, therefore, we recommend dissociation protocols that favor as little mechanical stress as possible. In our hands, enzymatic dissociation with 2–4 ml Accutase (Millipore) at 37°C for 3–5 minutes or light mechanical trituration through a 14-gauze needle (3–5 times) is sufficient to dissociate hNSC into single cells and small 2–6 cell clusters. Detailed methodology can be found elsewhere (Wakeman et al., 2009a,b).

If the EBs are exposed to 4°C while in suspension, IgM will make them clump in a virtually irreversible manner. In this case place the EBs in a 60 mm Petri dish and use curved scissors for eye surgery to cut the clumps in smaller pieces. Then resuspend EBs without dissociation and plate them as indicated in Figure 1 on matrigel rather than poly-D-lysine.

Anticipated Results

OLPs obtained by the isolation of NSC and their subsequent commitment to OLs utilizing this system are plated on anti-PSA-NCAM plates and will attain a bipolar morphology if maintained in freshly supplemented OSM. Cells can also be plated directly onto plastic (tissue culture grade). The morphology may look more flattened or "fibroblast-like", but if maintained in fresh OSM, the early markers such as Olig2, Tf, PDGF-R and NG2 will be expressed. At this stage, cells are still highly motile but will migrate less if plated onto poly-D-lysine. During this time, cells attain a more mature phenotype that truly represents their *in vivo* counterparts.

Each of our culture media formulations as previously described (Espinosa et al., 2002; 2009), have proven to be effective for the isolation, commitment and maintenance of NSCs and OLPs because they include the minimum and sufficient nutrients to support a given developmental stage in this lineage. Cultures at a given developmental stage could virtually be kept indefinitely. Nonetheless this is not possible only because the substrata used to coat the containers to enhance cell adhesion and well being for the cells, decays with time and therefore, cells cannot be kept indefinitely in these conditions. Because the substratum dictates the organization of the molecules on the cell membrane and the subsequent signal

transduction chain of events, the substratum of choice to isolate or to study the cells may yield slightly different results in terms of yield (i.e. OL numbers) (rev. Linnemann and Bock, 1989; Mauro et al., 1994).

Lastly, the needs and dynamics of a totipotent stem cell such as hiPS are much more numerous than a lineage restricted cell such as NSCs. The pace at which they develop is also much faster and therefore, lineage restriction needs to be aggressively, yet methodically performed to keep that pace at which these cells wish to develop. Keeping the finesse in the stoichiometry between reactants and products involved in lineage restriction is of the essence hence, the modifications performed to our chemically defined cell culture media to meet the requirements of hiPS to commit to the neural and OL phenotype. We believe that the levels of nutrients used in the current protocol have met the pace at which these cells are expected to develop.

Time Considerations

The initial dissection and preparation of the primary cell suspension takes approximately 2 h. From the moment cells are plated on anti-PSA-NCAM (if fed regularly with fresh humoral factors), 100 mm dishes can be confluent within 3–4 days. Thus, generating 20 vials of rat NSCs for cryostorage would take approximately 16 days. The generation of OLP from rNSC takes approximately 24 h; yet, generating OLP in high numbers (15 vials) for storage would take 4–6 weeks. Lineage progression of rat OL towards more mature phenotypes takes approximately 48 h in the specific culture medium (GDM or OLDEM). In addition, OL will still proliferate in GDM but at a much slower rate. Both, GDM and OLDEM media are favorable to protein synthesis but less favorable for cell proliferation.

Previously isolated ES cells and their NSC derivatives will need a longer period of time (approx. five times longer) to provide high numbers of NSC for frozen stocks. This time will vary depending on the origin of the sample. We have had similar success directing NSC from several species, utilizing the same chemically defined media; however, incubation times may need to be increased for full maturation in higher order mammals, such as primates. Induced cells loose NSC characteristics and acquire OLP features within 72 h, yet their cell cycle is much slower and therefore, it would be necessary to propagate these cells 8 to 10 weeks to be able to create a healthy stock (6 to 8 vials) of human (Hu) OLP. Previously established NSC lines (Snyder et al., 1992) can also be propagated and specified into the OL phenotype using the system described here.

As for the time needed to obtain adequate numbers of human OLs from hiPS now that we have standardized and optimized the culture system described here, it takes us around 8 weeks to obtain enough OLs derived from human embryonic brain using the method described in Figure 6 and 10. This is because cells need either to be isolated, propagated and then committed to OLs, or isolated and specified to the OL lineage and then propagated to obtain the desired yield. Both methods offer advantages in terms of the type of studies to be performed, i.e. disease models, or cell replacement therapies where the method of choice might be using hiPS as the starting material for transplantation. These cells may also be used in global gene and cell replacement strategies (Rev: Park 2002b)

Acknowledgments

Abbreviations

Oct4	octamer binding transcription factor 4
DNA	deoxyribonucleic acid
MS	Multiple Sclerosis
ROCK	Rho-associated protein kinase
SB431542	TGFbeta/Smad Inhibitor
O4	anti-sulfatides monoclonal antibody
Shh	sonic hedgehog
RA	retinoic acid
bFGF	basic fibroblast growth factor
EBs	embryoid bodies
MEF medium	Mouse embryonic fibroblast expansion and freezing medium
PS medium	
N2	A suplement containing: Recombinant human insulin Human transferrin (iron-saturated) Sodium selenite, Putrescine, Progesterone
EDTA	Ethylenediaminetetraacetic acid
KOSR	Knock Out Serum Replacement
KO-DMEM	DMEM supplemented with KO
IgM	Immunoglobulin M
GDM	glial defined culture medium
DMSO	Dimethyl Sulfoxide
OLSBN	OLS medium +B27 +N2
nM	nano mOLsar
pOLsy-D-lysine	Multi-pOLsymer of lysine
PSA-NCAM	pOLsysialic acid neural cell adhesion mOLsecule

PSA-NCAM IgM	ant-PSA-NCAM IgM
IGF-1	insulin growth factor 1
T3	Triiodothyronine
SYTOX	
STM	stem cell medium
STMc	stem cell medium supplemented with additives
Basal STM	stem medium without supplements
MTA	material transfer agreement
STM-II	stem culture medium alternative formula
OLSDEM	OLsigodendrocyte defined medium
STM-CM	stem conditioned medium
self-CM	self-conditioned medium
2-D cultures	bidimensional cultures
GC+/−	galactocerebrosides positive or negative
CNP+/−	2',3'-Cyclic-nucleotide 3'-phosphodiesterase positive or negative
ED14–16	embryonic day 14 to 15
CM	conditioned medium
RCF	relative centrifugal force
RMS	rostral migratory stream
P0–P5	Postnatal day 0 to 5
dPBS	distilled phosphate buffer
Et-OH	ethylic alcohOLs
SN	supernatant
FBS	Fetal Bovine Serum
EB medium	Embryoid Body Medium
NEAA	Non-Essential Amionacids
ITS	Insulin-Transferrin-Selenite
D-MEM	Dulbecco minimal essential medium

CNS	Central Nervous System
B104CM	B104 neuroblastoma cell-conditioned medium
hNSC	
Tf	transferrin
PDGF-R	Platelet-derived growth factor
NG2	neural/glial antigen 2
rNSC	rodent neural stem cell
Hu OLSP	uman OLsigodendrocytes
FACS	fluorescent activated flow sorting
MBP	myelin basic protein
NF-200	
HuNu	Human Nuclei marker
PAX6	Paired box protein Pax-6
RC2	anti-radial glia antibody
CC1	also known as APC anti-adenomatous pOLsyposis cOLsi protein
GFAP	glial fibrillary acidic protein
md	myelin-deficient
FB+	fluorescent fast blue positive
IS	implant site

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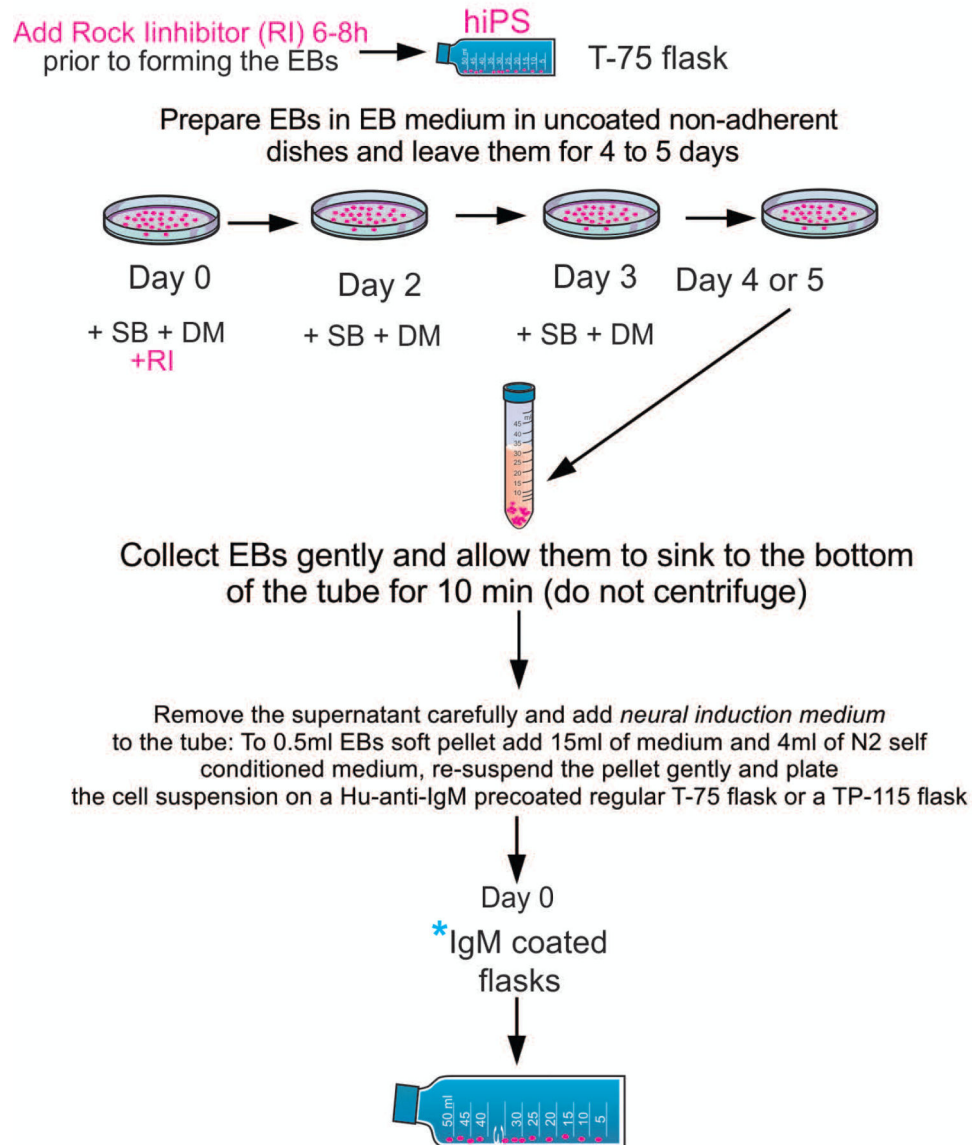
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From EBs to the Neural Lineage

This step entails the inhibition of mesoderm and endoderm with the concomitant initiation of induction of ectoderm to neural phenotype

Add Rock Iinhibitor (RI) 6-8h prior to forming the EBs → hiPS T-75 flask

Prepare EBs in EB medium in uncoated non-adherent dishes and leave them for 4 to 5 days

Day 0 → Day 2 → Day 3 → Day 4 or 5

+ SB + DM +SB + DM + SB + DM
+RI

Collect EBs gently and allow them to sink to the bottom of the tube for 10 min (do not centrifuge)

Remove the supernatant carefully and add *neural induction medium* to the tube: To 0.5ml EBs soft pellet add 15ml of medium and 4ml of N2 self conditioned medium, re-suspend the pellet gently and plate the cell suspension on a Hu-anti-IgM precoated regular T-75 flask or a TP-115 flask

Day 0
*IgM coated flasks

Figure 1.
Ectoderm selection can be performed by exposing hiPS to SB431542 (SB) and dorsomorphin (DM), which instruct ectoderm formation preferentially, inhibiting endodermal and mesodermal commitment. All reagents should be freshly prepared as the stock solutions decay fast (between 1 [SB] and 3 [DM] months). Thus, it is suggested to create a stock of hiPS-derived OL while the reagents are fresh. For the procedures described here, the coating of choice was IgM. Nonetheless, if the EBs have been exposed to 4°C

while in suspension they would clump together and the vast majority will not adhere to the IgM-coated surface. In this case it is imperative to use Matrigel or poly-D-lysine.
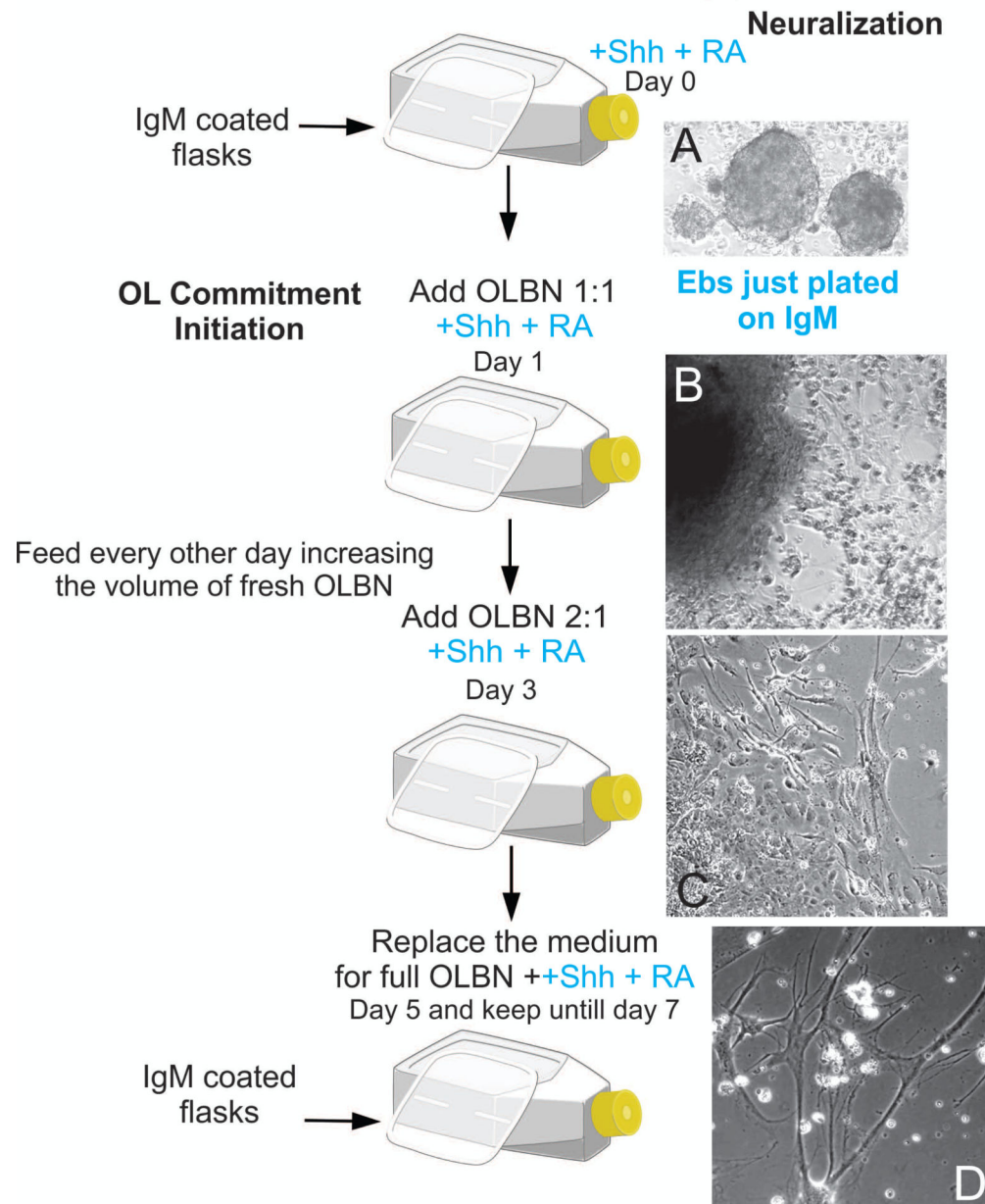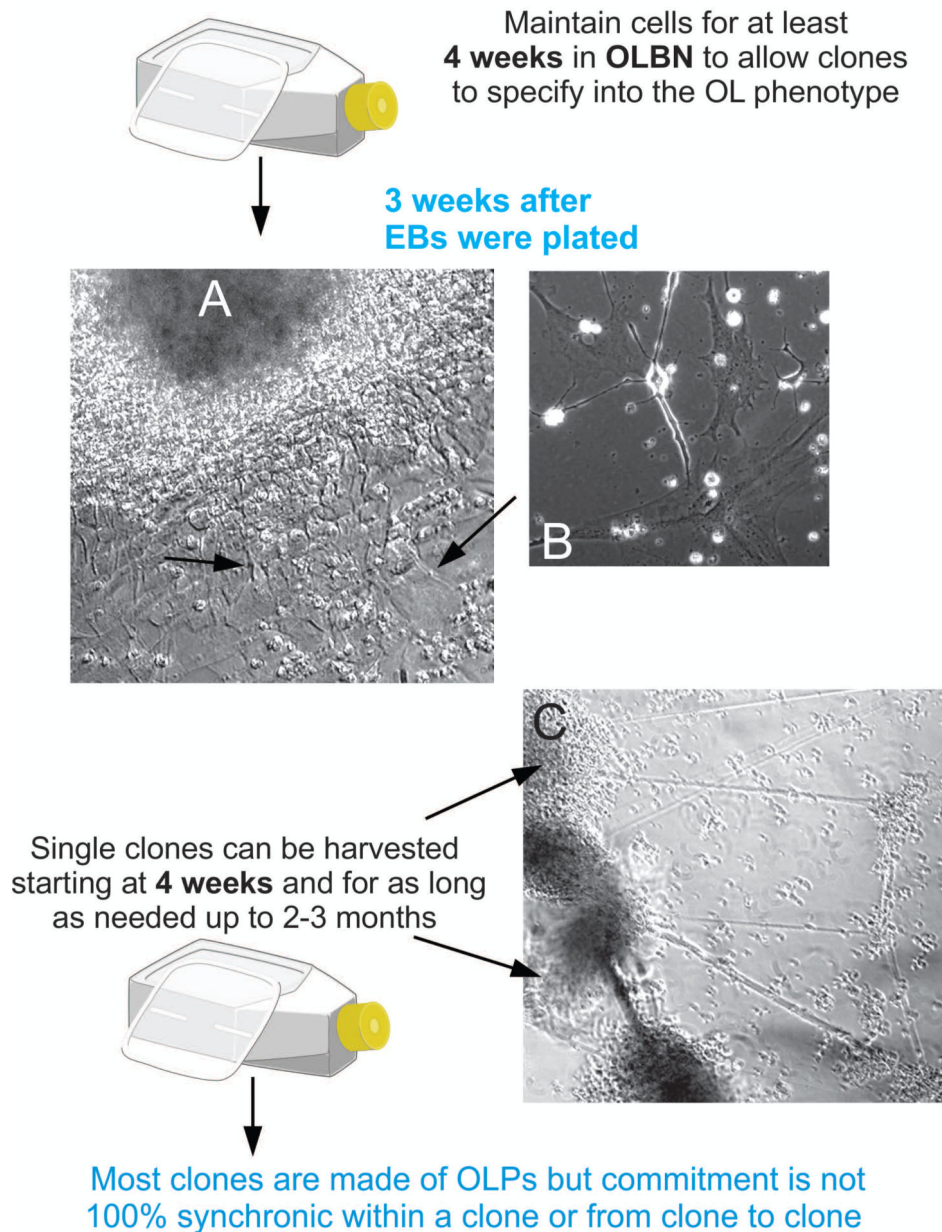
Figure 2.
Once the EBs are transferred to the IgM-coated flask, the culture medium to favor neuralization is the N2 medium, which properties will be enhanced by Shh and RA. A sustained level of these two components leaves virtually no room for other cell types present in the cultures (i.e. flat, fibroblast-like cells, panel C) to prosper. Bi-refringent cells populate both the substratum and flat cells that are often used as anchorage zones (panel D).

Ols specification and migration

Maintain cells for at least **4 weeks** in **OLBN** to allow clones to specify into the OL phenotype

3 weeks after EBs were plated

Single clones can be harvested starting at **4 weeks** and for as long as needed up to 2-3 months

Most clones are made of OLPs but commitment is not 100% synchronic within a clone or from clone to clone

Figure 3.
With time in OLBN, flat cells tend to disappear and numerous cells migrate away from the mother clone (panels A and B). These bipolar cells extend their processes to migrate or to connect with other cells while serving as bridges for other cells to migrate on their extensions (panel C). As time in culture increases, cells tend to migrate using other cell processes rather than the substratum.

Transferring clones to a desired container and/or OL stage specific medium

Carefully aspirate rich and round clones with blue tip or a pre-sterilized Pasteur pipette without touching or scratching the cells adhered to the substratum in direct contact with the flask surface
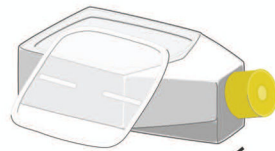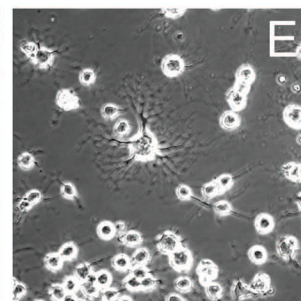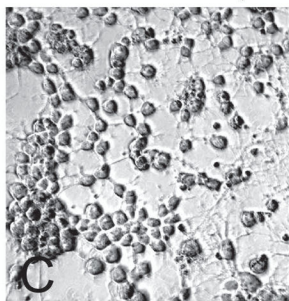
Clones will continue to grow across for at least 2 months and can be picked up at various time points as needed
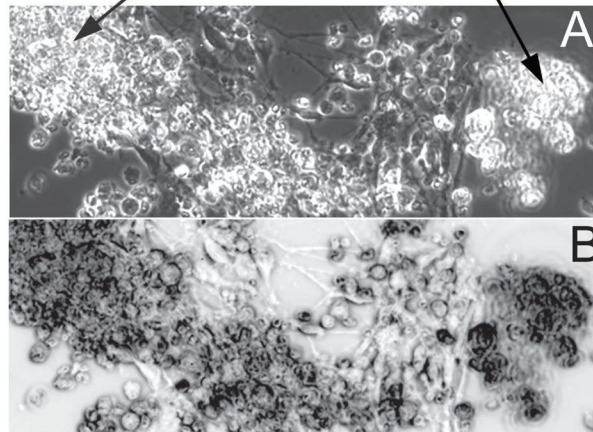
View of cells 2d after replated **P1**   **P2**, 2d on BS1   **P2**, 4d on BS1

Figure 4.
Transferring Clones successfully. The main aspect to consider is not to scratch the substratum with the yellow tip, aspirating a clone carefully will allow flat cells to remain in the mother flask while bringing the entire clone of round, bright cells to the new cell culture container (panel A). OL will proliferate and arrange as small clones, as well as single cells in a very homogeneous looking culture (panel C). With this cell culture medium-based selection method, there is no need to use FACS to enrich the cultures in OLP cells, and therefore, the cells are healthy and yield large numbers. Cells can be re-plated (transferred to

new culture containers) using the same stage specific medium, or the next stage specific medium such as BS1 (panel D). With time, cells tend to become multipolar (panel E).
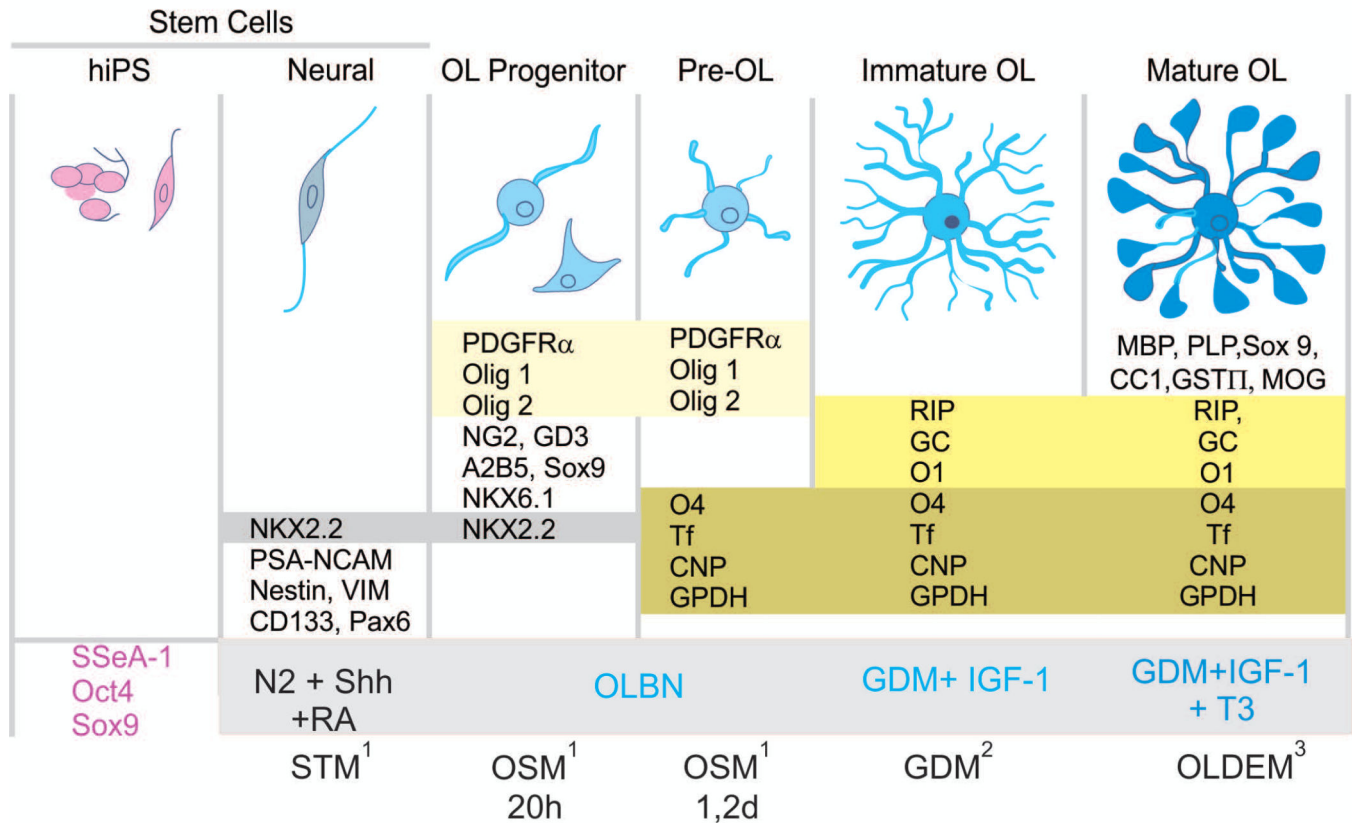
hiPS derived Oligodendrocyte Specification by Instruction

Stem Cells		OL Progenitor	Pre-OL	Immature OL	Mature OL
hiPS	Neural				

		OL Progenitor	Pre-OL	Immature OL	Mature OL
		PDGFRα Olig 1 Olig 2 NG2, GD3 A2B5, Sox9 NKX6.1	PDGFRα Olig 1 Olig 2		MBP, PLP,Sox 9, CC1,GSTΠ, MOG
				RIP GC O1	RIP, GC O1
	NKX2.2	NKX2.2			
	PSA-NCAM Nestin, VIM CD133, Pax6		O4 Tf CNP GPDH	O4 Tf CNP GPDH	O4 Tf CNP GPDH

SSeA-1 Oct4 Sox9	N2 + Shh +RA	OLBN		GDM+ IGF-1	GDM+IGF-1 + T3
	STM[1]	OSM[1] 20h	OSM[1] 1,2d	GDM[2]	OLDEM[3]

[1]STM, OSM (Espinosa et al., 2002), [2]GDM (Espinosa et al., 1994), [3]OLDEM (Espinosa et al., 1988, 1997)

Chart modified from Espinosa et al., 2009.

Figure 5. hiPS-derived OL specification by instruction through the specific culture medium
Human pluripotent stem cells can be obtained from skin fibroblasts, by introducing the 4 transcription factors described by Takahashi and Yamanaka (2006). Then, the components of the culture medium direct and determine the fate of hiPS. Our culture medium previously designed to favor the OL phenotype when starting from neural stem cells (Espinosa et al., 2002), needed to be enriched with the specific molecules present during the development of the CNS to successfully direct human induced pluripotent stem cells to the neural and OL phenotype (see pink highlighted area). Once this transition has been accomplished, OL undergo sequential morphological changes from OLP and acquire characteristics inherent to a functional OL. A partial list of OL markers below each developmental stage is not exhaustive but represents frequently used markers to identify OLs and their specific developmental stage. The main difference between the culture medium for lineage progression and maintenance of hiPS-derived OL is that they need N2 while in the progenitor stage, and IGF-1 + T3 when a mature phenotype is desired. When starting cultures from brain-derived neural stem cells, we have previously described the neural stem cell medium (STM; Espinosa-Jeffrey et al., 2002); OL specification medium (OSM; Espinosa-Jeffrey et al., 2002); glia defined medium (GDM; Espinosa de los Monteros and de

Vellis, 1996); OLDEM (Espinosa de los Monteros et al,1988, 1997). Chart Modified from (Espinosa-Jeffrey et al., 2009).
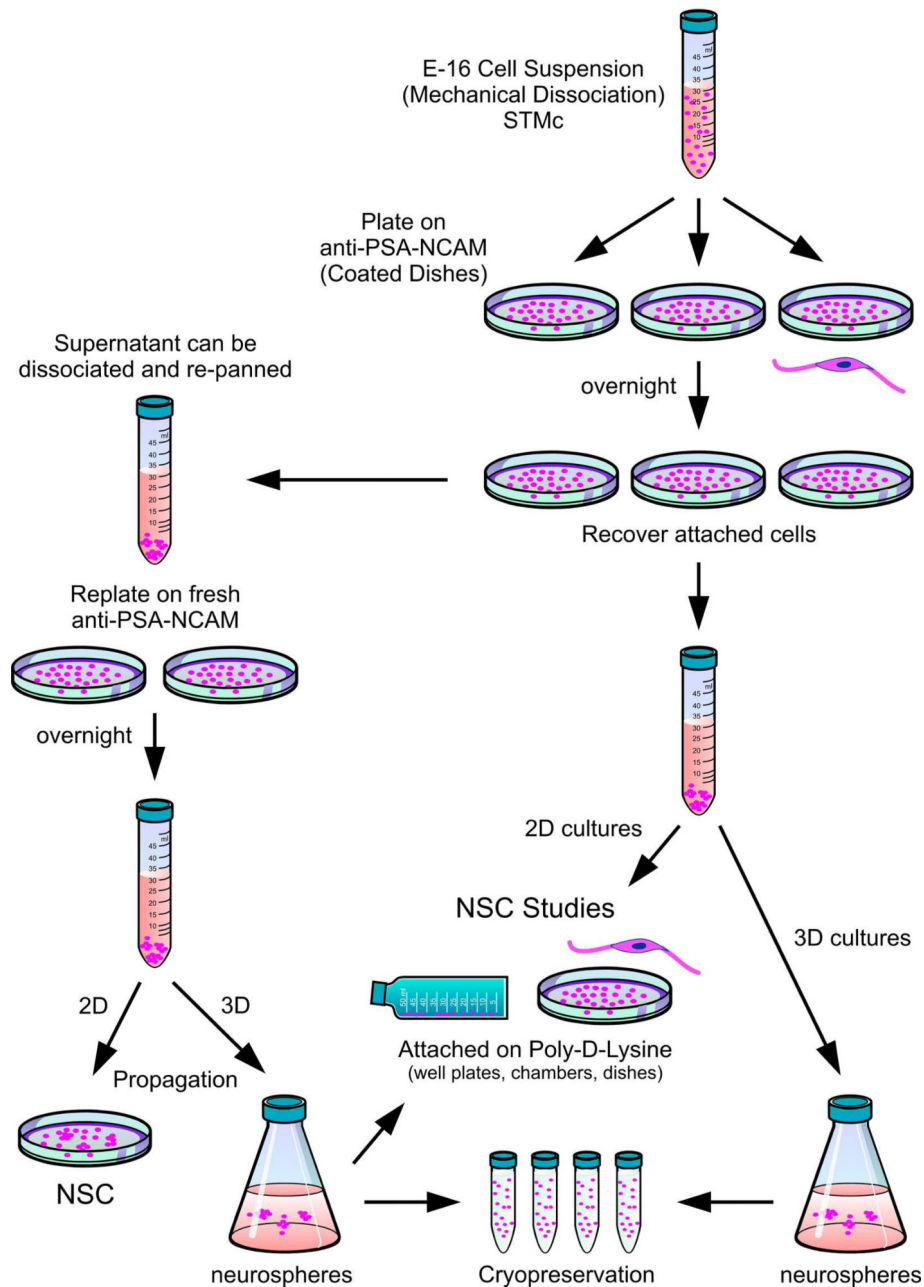
Rodent Neural Stem Cell Preparation

Figure 6. Rodent neural stem cell preparation

Following dissection, the cell suspension is plated on anti-PSA-NCAM antibody–coated dishes and allowed to adhere. The process can be performed repeatedly to increase the numbers of neural stem cells, as two-dimensional cultures or three-dimensional "sphere" cultures (shown on the left side of the diagram). Every time cells are propagated, use anti-PSA-NCAM-coated dishes. Alternatively, cells can be propagated and immediately used for cell culture experiments (as shown on the right side of the diagram). While we prefer to use

committed OL progenitors for cell transplants, other investigators also use uncommitted progenitors for grafting.
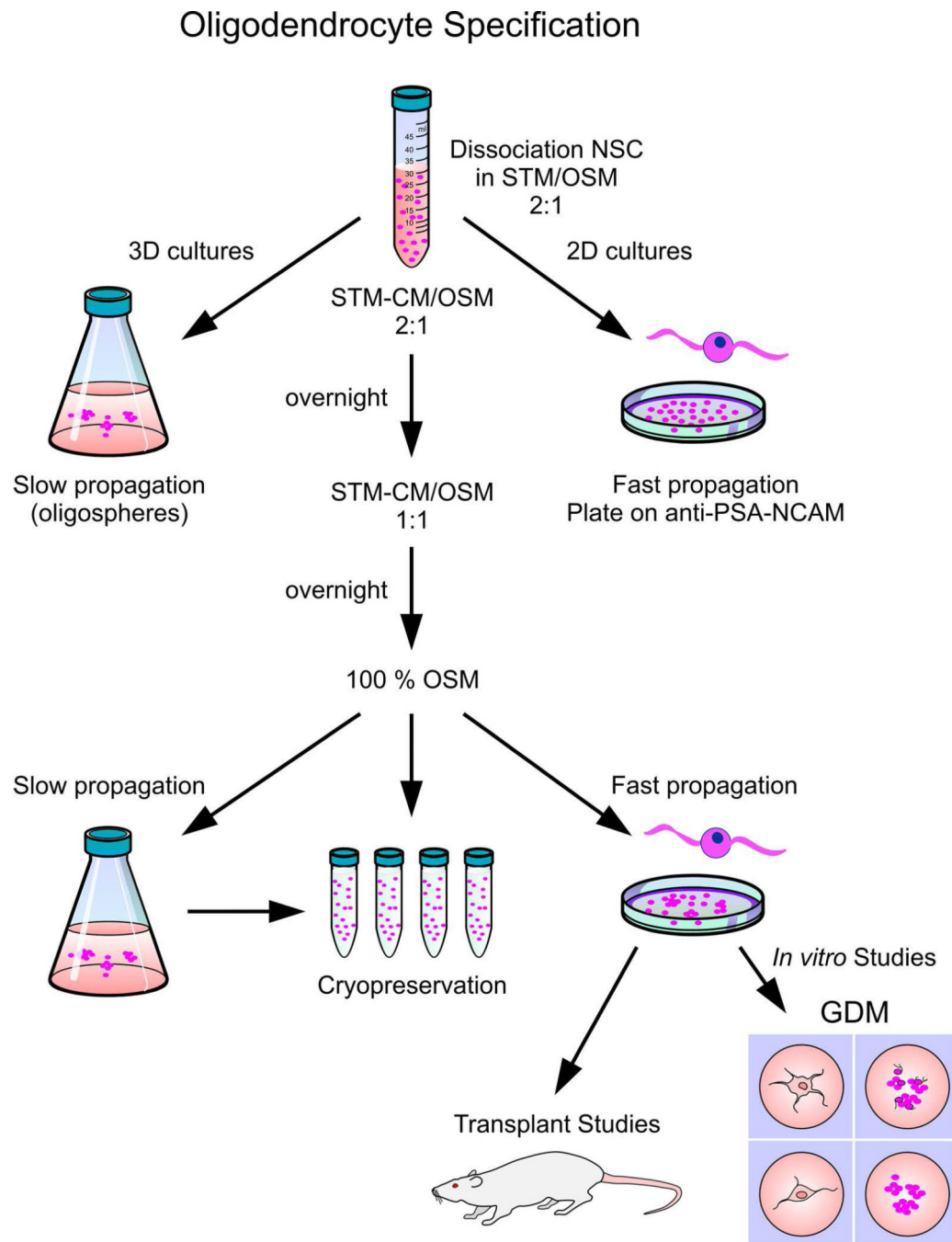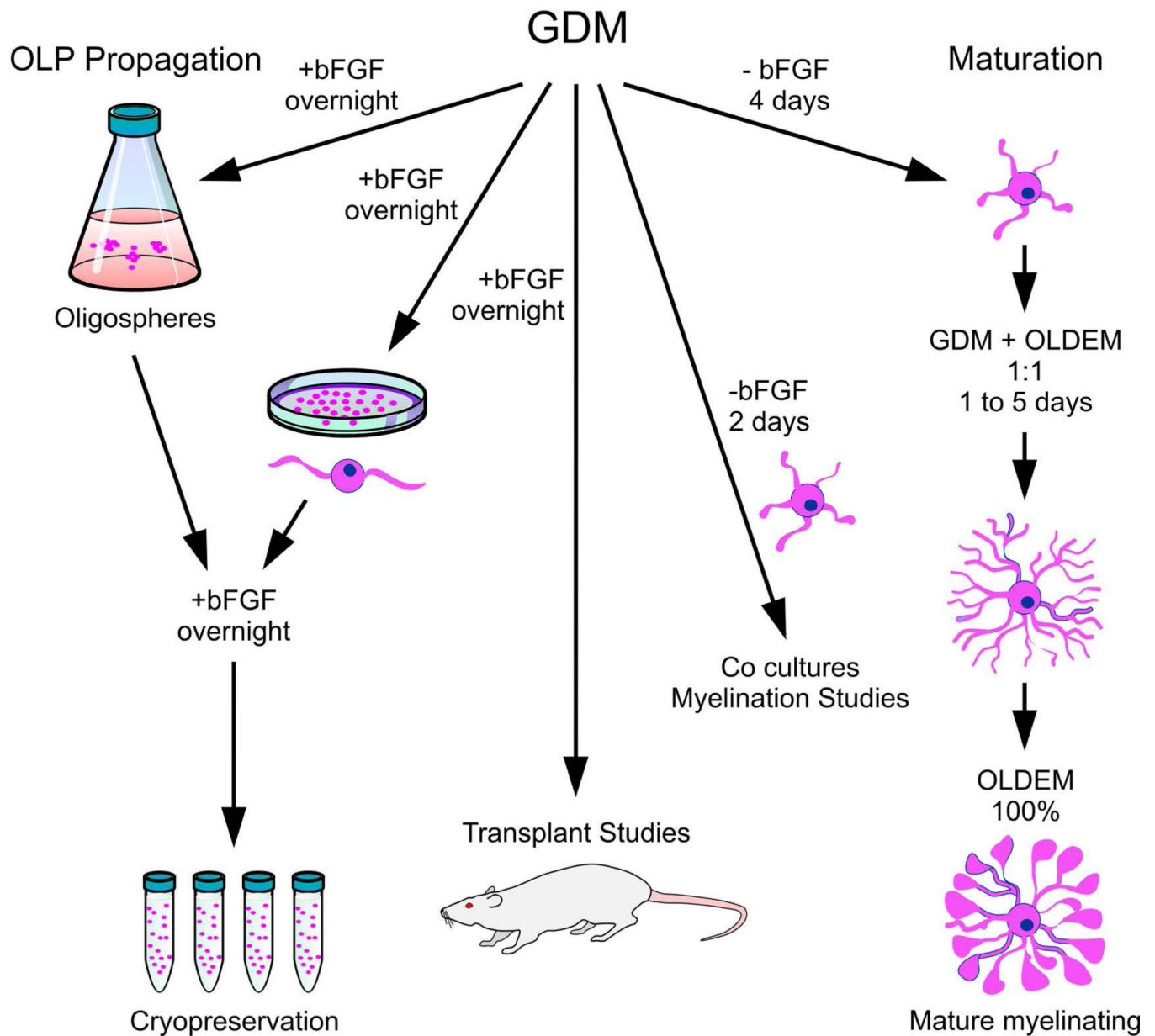
Figure 7.

OL specification by instruction. The transition from NSC to committed OL lineage is brief, but sequential rather than abrupt. In order for cells to survive, they must acclimate to their new environment. OLP can be propagated to create frozen stocks as three-dimensional "oligosphere" cultures (shown on the left of the diagram), or frozen without propagation (as shown in the sequence in the center of the diagram). OLP can also be propagated in two-dimensional cultures for cryopreservation, for specific cell culture experiments, or for cell replacement therapies (as shown on the right side of the diagram).

Oligodendrocyte Lineage Progression and Maturation

Figure 8.

OL lineage progression and maturation. After commitment of NSC to the OL lineage, cells are propagated at the OLP stage to create a frozen stock (steps indicated on the left portion of the flow chart) or processed further for transplantation studies (as shown by the middle arrow on the diagram). To allow OLP to further mature along the OL lineage and become myelinating cells, OLP cell cultures are transitioned into OLDEM for at least 48 h. Once OL have reached this stage of maturation, they are excellent for cell culture studies but are not recommended for cell grafting. Detachment from the substrate can damage the numerous
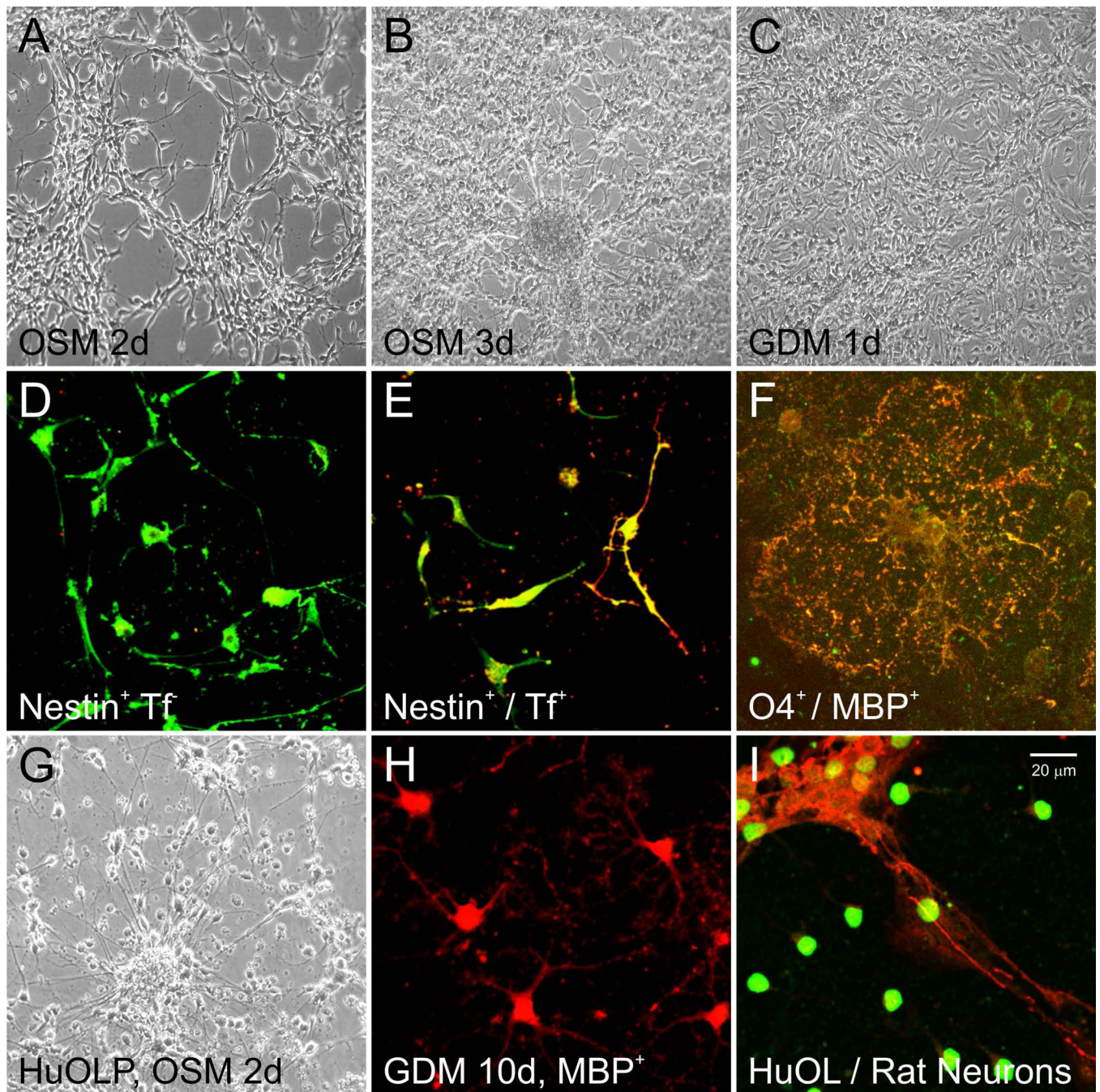
delicate cell processes, therefore these cultures are no longer a quality source for cell transplants.

Figure 9.
Phase contrast view of neural stem cells derived from embryonic day 16 rat brain at passage number 2 (P2). NSC were plated and maintained in OSM for 2 days (A), 3 days (B), or 3 days in OSM then switched to GDM for 1 day (C). Cells still proliferate while in OSM. When cells from (A or B) are plated and maintained in OSM on poly-D-lysine-coated coverslips for 1 day, they start to display a bipolar or multipolar morphology (D), and most express the immature precursor marker, nestin (green) but not Tf (red), an early marker for OL. After 2 days in OSM, bipolar nestin+ cells co-express transferrin (Tf) (E). After 4 days

in OSM, cells were switched to GDM for 1 day; they developed numerous cell processes and co-expressed sulfatides (recognized by the anti-O4 antibody, green) and myelin basic protein (MBP; red) (F). Panels G to I are human cells. (G) Phase-contrast view of human NSCs (HFB-2050) acclimated and expanded in STM, then replated and maintained in OSM for 2 days. (H) OL derived from human NSCs (HFB-2050) were specified to the OL lineage with OSM and maintained in GDM for 10 days. OL matured and started to express MBP (red). (I) Rat cortical neurons (NFM-200-red) were cultured for 10 days, then human OLP derived from NSC (HFB-2050) were added in co-culture for 24 hr. These cells were labeled with human nuclei marker (HuNu, green).
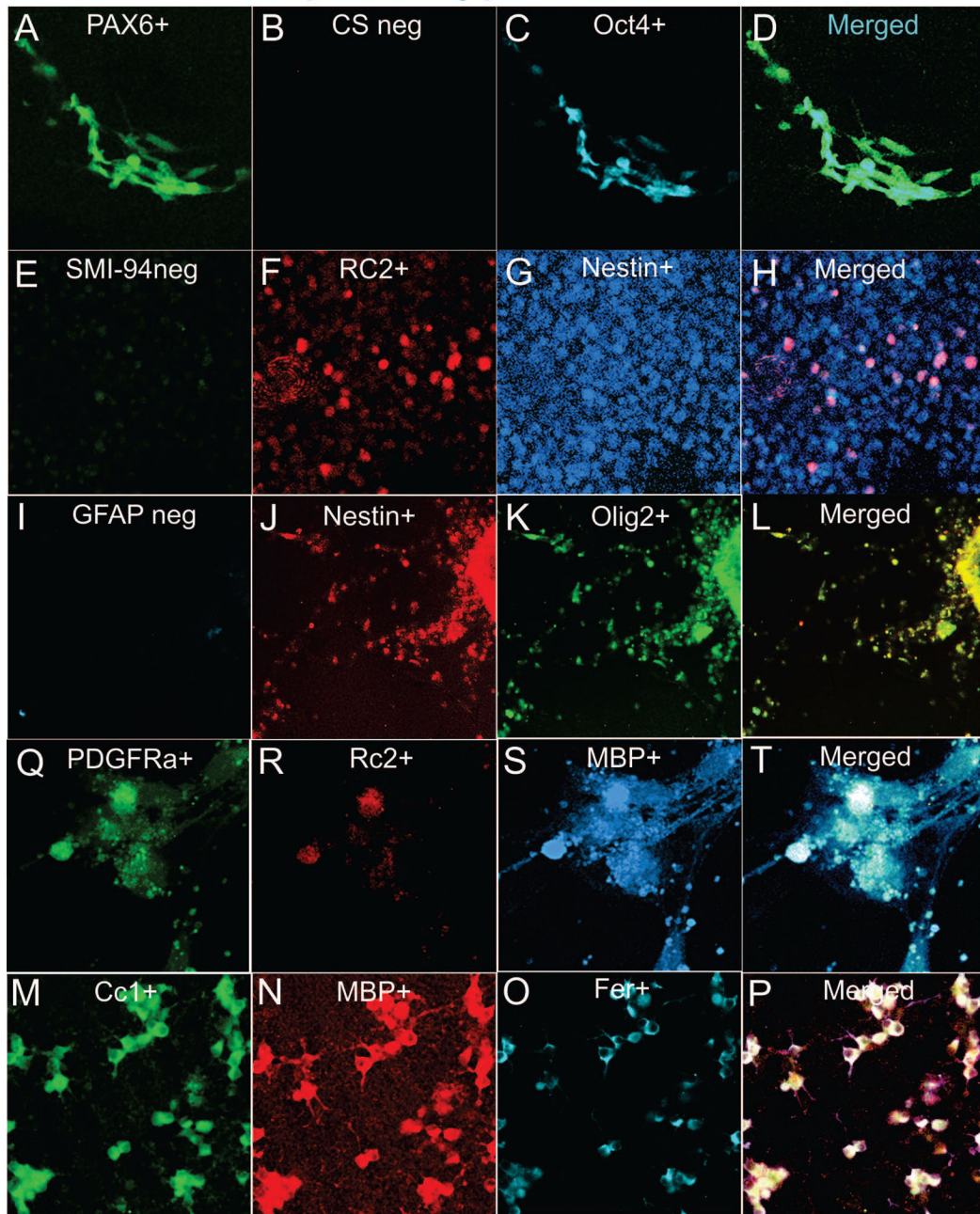
Figure 10. hiPS derived OL express neural progenitor and OL cell lineage and stage specific markers recapitulating their development *in vivo*

Two days after EB's were plated cells express

PAX6

and

OCT4

(**panels A–D**; *developmental stage corresponding to* Figure 2 *panel B*). By day 6 (**panels F–H**) expression of

RC2

a marker for radial glia (F) at different intensity could be observed with a concomitant expression of

nestin

(**H**) by virtually all cells (panel G), and not all nestin + cells bared the RC2 staining (stage corresponding to Figure 2 panel C). The co-expression of

nestin

and

Olig2

can be observed within the next three weeks (**panels J–L**) most nestin expressing cells co-express Olig2 (panel L; *developmental stage corresponding to* Figure 2 *panel C*). When clones are harvested 4 weeks after starting their fate specification and re-plated on poly-D-Lysine in BS1 and allowed to become mature i.e. 6 days after seeding (**panels M–P**) cells expressing faintly

PDGFRa

(M) in their extended membrane and a few cells still bearing it in their cell body, they appear to be losing the RC2 staining (N) while intensely expressing

MBP

in the cell body as well as, in their extended membrane sheath (O) the colocalization of markers is shown in panel (P). OLs in these cultures also express intensely in their cell body and immediate processes the mature markers
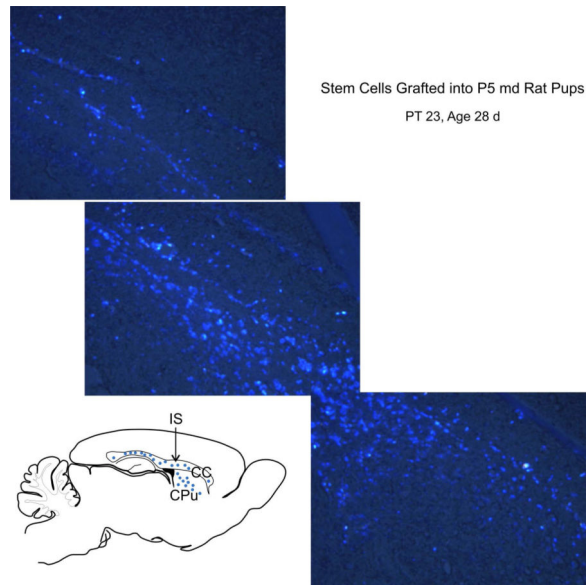
CC1

and

MBP

as well as

ferritin

(panels Q–R). Panel B shows control serum for Alexa-fluor 408,540 and 633. Panel I shows immunoreactivity for

GFAP

is absent from these cultures.

Figure 11.
Human OL derived from (HFB-2050) human fetal NSC were labeled with fluorescent fast blue (FB; Sigma, cat. no. F-5756). A total of 60,000 cells were grafted into the corpus callosum (CC) of P(5) rat pups born to a myelin-deficient (md) carrier mother. At 23 days after grafting, samples were harvested and examined. Grafted NSC survived and migrated extensively within the host brain parenchyma extending along the corpus callosum (CC) and caudate putamen (CPu). In the sketch, dots represent the location where FB+ cells were found. The sketch represents a sagittal view of the transplanted rat brain at 28 days of age, IS indicates where cells were implanted.

Table 1

Reagents and Materials for Culture Media, Cell Growth and Cryopreservation

#	Name	Company	Catalog #
1	Sodium Selenium	DIFCO	0608-15
2	Insulin	Sigma	1-5500
3	Human Transferrin	Sigma	T-2252
4	Putrescine	Sigma	P-7505
5	Sodium Bicarbonate	Fisher	S233-500
6	Sodium Selenite	Sigma	S5261
7	Bovine Serum Albumin	Sigma	A3156
8	B-27 Supplement	Invitrogen	17504-044
9	D-MEM/ F12 medium	GIBCO BRL	12100-046
10	D-MEM low glucose medium	GIBCO BRL	11054-020
11	Anti-PSA-NCAM IgM	Iowa DSHB	http://www.uiowa.edu/~dshbwww/
12	Recombinant Human EGF	Invitrogen	Cat.132476-051
13	Recombinant Human bFGF	Invitrogen	Cat.13256-029
14	Tris	Fisher	BP 152-1
15	D(+) galactose	Sigma	G0625
16	Kanamycin	Sigma	K0254
17	12.5, 75 cm^2 T-Flasks	Falcon	353003
18	Petri dishes (Non-TC)	Falcon	351029
19	SYTOX	Invitrogen	S7020
20	Creatine	Sigma	C3630-100G
21	Tissue Culture Test tubes	Fisher	14-956-6A
22	5ml sterile pipettes	Falcon	357543
23	Cell strainers	BD Falcon	REF. 352350
24	12ml Syringes sterile	Tyco Healthcare	512852
25	20 ml Syringes sterile	Jone Kendall	520673
26	Cryo-Tube Vials	NUNC	366656
27	Cryogenic Freezing container	NALGENE	EW-44400-00
28	Neurobasal Medium	GIBCO	21103-049
29	B-27 Supp. without Vitamin A	GIBCO	12587-010
30	GlutaMAX	GIBCO	35050-061
31	Heparin	Sigma	H-3149
32	Normocin	InVivoGen	Ant-nr-1
33	Leukemia Inhibitory Factor	Millipore	LIF-1010
34	Fluorescent Fast Blue	Sigma	F5756
35	Progesterone	Sigma	P7556
36	Normocin		
37	KnockOut DMEM	Gibco-BRL	10829-018

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#	Name	Company	Catalog #
38	KnockOut serum replacement	GIBCO BRL	10828
39	Retinoic acid	Sigma	R2625
40	Accutase	Innov. Cell Tech.	AT104
41	Tissue Culture Flask, T115 w/ recloseable lid	TPP	TP90652
45	Anti-IgM whole Molecule		