Video Article

Progenitor-derived Oligodendrocyte Culture System from Human Fetal Brain

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Abstract

Differentiation of human neural progenitors into neuronal and glial cell types offers a model to study and compare molecular regulation of neural cell lineage development. *In vitro* expansion of neural progenitors from fetal CNS tissue has been well characterized. Despite the identification and isolation of glial progenitors from adult human sub-cortical white matter and development of various culture conditions to direct differentiation of fetal neural progenitors into myelin producing oligodendrocytes, acquiring sufficient human oligodendrocytes for *in vitro* experimentation remains difficult. Differentiation of galactocerebroside^{+} (GalC) and O4^{+} oligodendrocyte precursor or progenitor cells (OPC) from neural precursor cells has been reported using second trimester fetal brain. However, these cells do not proliferate in the absence of support cells including astrocytes and neurons, and are lost quickly over time in culture. The need remains for a culture system to produce cells of the oligodendrocyte lineage suitable for *in vitro* experimentation.

Culture of primary human oligodendrocytes could, for example, be a useful model to study the pathogenesis of neurotropic infectious agents like the human polyomavirus, JCV, that *in vivo* infects those cells. These cultured cells could also provide models of other demyelinating diseases of the central nervous system (CNS). Primary, human fetal brain-derived, multipotential neural progenitor cells proliferate *in vitro* while maintaining the capacity to differentiate into neurons (progenitor-derived neurons, PDN) and astrocytes (progenitor-derived astrocytes, PDA) This study shows that neural progenitors can be induced to differentiate through many of the stages of oligodendrocytic lineage development (progenitor-derived oligodendrocytes, PDO). We culture neural progenitor cells in DMEM-F12 serum-free media supplemented with basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF-AA), Sonic hedgehog (Shh), neurotrophic factor 3 (NT-3), N-2 and triiodothyronine (T3). The cultured cells are passaged at 2.5e6 cells per 75cm flasks approximately every seven days. Using these conditions, the majority of the cells in culture maintain a morphology characterized by few processes and express markers of pre-oligodendrocyte cells, such as A2B5 and O-4. When we remove the four growth factors (GF) (bFGF, PDGF-AA, Shh, NT-3) and add conditioned media from PDN, the cells start to acquire more processes and express markers specific of oligodendrocyte differentiation, such as GalC and myelin basic protein (MBP). We performed phenotypic characterization using multicolor flow cytometry to identify unique markers of oligodendrocyte.

Video Link

The video component of this article can be found at http://www.jove.com/video/4274/

Protocol

Note: For routine culturing of neural progenitor and oligodendrocytic lineage cells, incubation is done at 37 °C in a humidified 5% CO_2 atmosphere. Every 2 days, the medium is replaced using 50 to 100% of fresh medium if culture is 40-70% confluent. At the time of near confluency, the cultures are passaged at 2-2.5e6/T75 flask usually on a weekly schedule.

1. Preparing the Coated Flask

1. To prepare coated flasks dilute 5 mg of poly-D-lysine (PDL) in 100 ml of deionized water (DI-water), then coat T75 flasks with 50 µg/ml of PDL at room temperature (RT) in the dark. After 1½ hr, aspirate the PDL and rinse the flasks once with DI-water. Let the flasks dry before seeding the cells (**Table 1**).

2. Starting Differentiation of Neural Progenitor Cells into Progenitor-derived Oligodendrocytes (PDO)

The protocol to isolate human CNS progenitor cells is described in a previous publication and it is not part of this protocol[1]. Human CNS progenitor cells were isolated from the telencephalon of an 8-week gestation fetal brain, obtained in accordance with NIH guidelines.

1. Grow multipotential population of neural progenitor cells on PDL coated flasks in neurobasal medium supplemented with 25 ng/ml of bFGF and 20 ng/ml epidermal growth factor (EGF) (**Figure 1A**). Do not use them if higher than passage 11 (p11).
2. Start to differentiate the neural progenitor cells when the culture is approximately 70% confluent.
3. Make complete differentiation oligodendrocyte medium using serum-free DMEM/HAMS F12 1:1 medium supplemented with bovine serum albumin, L-glutamine, gentamicin, N2 components, T3, Shh, NT-3, bFGF, and PDGF-AA (**Table 1**). This medium will be defined as oligo medium with growth factors (oligo medium + GF).
4. Remove progenitor media from the flasks, rinse cells once with phosphate-buffered saline (PBS), then add oligo medium + GF (details described in 2.3) to start oligodendrocyte differentiation. Only cells committed to the oligodendrocytic lineage will grow (**Figure 1B**). Culture the cells in this medium for 3 weeks.
5. Every week, when the cells are at 90-95% confluence, passage them using trypsin (0.05%)-EDTA (0.1%) (4 ml for a T75 flask). Transfer the entire medium from the cells to be passaged into a 50 ml conical tube; this conditioned medium will be used to quench the trypsin. Make sure not to dry out the cells. Then add trypsin.
6. Incubate cells in trypsin for 5 min at RT, gently tapping the flask several times to help cell detachment.
7. Quench the trypsin by adding 4-5 ml of conditioned medium to the flask with the detached cells.
8. Transfer the medium and the cells to the same 50 ml conical tube. Centrifuge the medium and the cells at 1,200 rpm (~300 x g) for 5 min at RT.
9. Aspirate the supernatant, resuspend the cell pellet, gently tipping the tube, and then add fresh oligo medium + GF . Gently pipette medium and cells up and down to make a uniform cell suspension.
10. Count the cells, and transfer 2.5e6 into each new PDL-coated T75 flask.

3. Final Step in the Differentiation of Oligodendrocytes

1. After 3 weeks of culture in oligo medium + GF, when the cells are at 70-80% confluence, start the final process of differentiation (**Figure 1C**).
2. Prepare medium in the same way as the oligo medium + GF but without adding the four GF: Shh, NT-3, bFGF and PDGF-AA. This medium without the four GF will be defined as oligo medium - GF.
3. Aspirate the oligo medium + GF from the flask, rinse once with PBS and then add a total volume of 16 ml of medium, of which ¾ (12 ml) is oligo medium - GF and ¼ (4 ml) is PDN conditioned medium, for 6-10 days). The use of conditioned medium from PDN is not critical to the differentiation process or to the survival of the cells. We in fact observed that only direct contact of PDO with PDN cells in co-culture experiments had an effect on the length of the PDO survival. The survival time in oligo medium - GF could be prolonged to two or three weeks if the PDO cells are co-cultured with PDN cells. The protocol to differentiate human neural progenitor cells into PDN is described in a previous publication and it is not part of this protocol[1]. Growth factor withdrawal from the culture medium results in progressively differentiated culture of cells with multiple processes (**Figure 1D**).

4. Flow Cytometry Assay

The flow cytometry assay compares the acquisition of oligodendrocyte markers during the differentiation process in relationship with those of their parental population.

1. Use neural progenitors as control. Seed PDO cells at $2.5x10^6$ cells in two T75 flasks coated with poly-D-lysine in oligo medium + GF.
2. A week later, replace medium with fresh oligo medium + GF in flasks as a control culture for cell viability, while other flasks had oligo medium - GF.
3. To dissociate PDO cells in both media use 20 U/ml of papain and not trypsin. This is because papain has a gentler effect on the cells during the dissociation preserving the cellular morphology.
4. Add 5 ml of Earle's Balanced Salt Solution (EBSS) to a papain vial. Place the vial at 37 °C for 10 min or until the papain is completely dissolved and the solution appears clear. This is the papain solution used to dissociate the cells.
5. Add 0.5 ml of EBSS to a Deoxyribonuclease I (DNase) vial (**Table 1**). Mix gently. Add 0.25 ml of this solution to the vial of dissolved papain. This preparation contains a final concentration of approximately 20 U/ml papain and 0.005% DNase.
6. Place papain solution (as described above) on the cells and incubate at 37 °C for 15-30 min; monitor the detachment of the cells using a microscope. Stop the reaction with 5 ml of medium with or without GF and centrifuge at 1,200 rpm for 7 min.
7. Transfer cell pellets from both flasks into two different 5 ml polypropylene tubes and wash them with normal physiological medium (NPM: 145 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 10 mM Hepes and 10 mM glucose) supplemented with 1 mg/ml bovine serum albumin (NPM+BSA).
8. Perform surface immunostaining at 4 °C for 20 min using specific and appropriately titrated primary antibodies for A2B5, O4, GalC (**Table 2**).
9. Wash cells with NPM+BSA at 4 °C for 5 min and incubate them with specific secondary antibodies at 4 °C for 20 min (**Table 2**).
10. To stain intracellular antigens including nestin, glial fibrillary acidic protein (GFAP), class III β-tubulin, and myelin basic protein (MBP) (**Table 2**), fix cells in 2% paraformaldehyde (PFA) for 20 min at RT and permeabilize with cold 70% ethanol for 15 min at -20 °C.
11. Wash cells with 1x PBS and incubate with specific secondary antibodies (**Table 2**).
12. To discriminate between intact cells and subcellular debris during flow cytometry analysis, stain cells with 4,6-diamidino-2-phenyllindole, dihydrochloride (DAPI) (Life Technologies, Grand Island, NY).
13. In optimizing the above protocol, we performed all the appropriate control experiments to confirm the specificity of each immunoreagent. Briefly, for directly labeled antibodies, the appropriate negative control was a directly conjugated antibody of the same immunoglobulin isotype class (or subclass) and fluorochrome conjugate as the primary antibody (*i.e.*, the isotype control). For indirect immunostaining of

primary antibodies, the appropriate negative control was the secondary antibody conjugated to the fluorochrome of interest that specifically targeted the immunoglobulin class (or subclass) of the host in which the primary antibody was generated. When primary antibodies from multiple hosts (mouse, rabbit, chicken) were used together, we used the secondary antibody targeting the primary antibody of each host and each secondary antibody used was typically cross-adsorbed against the immunoglobulins from the other hosts to minimize cross-host immunoreactivity. The positive controls included direct or indirect immunostaining of cell preparations known to express the antigens of interest using either direct or indirect immunoreactions with the primary antibodies specific for these antigens. The above optimization methods were carried out once for each type of immunostaining experiments. Control immunoreactions revealed no significant cross-epitope immunoreactivity among primary and secondary antibodies. Flow cytometry was performed on uniformly suspended fluorescently labeled cells using a FACVantage SE cell sorter (BD Biosciences, San Jose, CA) equipped with three lasers, which provide excitation wavelengths tuned to 488 nm, 647 nm, and a broad UV (351-364 nm) (**Figure 2**).

5. Immunofluorescence Assay

Note: For immunofluorescence experiments, PDO are plated in oligo medium + GF or oligo medium - GF at 2.5e5 in 6 well plates coated with PDL. The cells are fixed in 2% PFA at various time points after withdrawal of growth factors . The antibody stain is performed on plastic 6 well plates instead of glass coverslips.

1. Remove media and add the 2% PFA. Incubate for 10 min at RT. Discard PFA. Wash cells 3 times with PBS, 5 min each time. Be careful not to dry out the cells.
2. Permeabilize cells for intracellular markers using 0.25% triton solution in PBS for 10 min at RT.
3. To prevent non-specific binding, block cells for 10 min with HHG (1 mM HEPES buffer, 2% horse serum, and 10% goat serum in Hanks' balanced salt solution). Dilute specific primary antibodies (**Table 2**) to a pre-determined concentration in HHG. Place diluted antibodies onto cells. Incubate 1 hr at RT on the shaker or at 4 °C overnight.
4. Wash cells 3 times with PBS, for 5 min each time. Dilute appropriate secondary antibodies (**Table 2**) coupled with a fluorochrome to a pre-determined concentration in HHG. Place the mixture of diluted secondary antibodies onto cells. Incubate 1 hr at RT on a shaker in the dark.
5. Wash cells 3 times with PBS, for 5 min each time.
6. To avoid photobleaching add 10 µl of Prolong Gold with DAPI to the cells and place a glass coverslip on top of them. Visualize labeled cells using an Axiovert 200M fluorescence microscope (Zeiss, Thornwood, NY) (**Figure 3**).

Representative Results

It is very important to start the differentiation process from a 70%-80% confluent neural progenitor cell culture (**Figure 1A**). Many cells will die out after changing the culture medium from progenitor to oligo medium since it includes specific growth factors. This indicates that the growth of neural progenitor cells not committed to an oligodendrocytic phenotype will not be supported by the new medium (**Figure 1B**). Incubation in oligo medium + GF for one week resulted in an intermediate culture exhibiting a narrow, bipolar morphology (**Figure 1B**). The cells are kept in oligo medium + GF for 3-4 weeks (**Figure 1C**). Growth factor withdrawal from the culture medium resulted in progressively differentiated culture of cells with multiple processes (**Figure 1D**). Flow cytometry is used for quantitative representation of the data.

Figures 2A, 2B and 2C demonstrate that the majority of neural progenitors expressed the neuroepithelial stem cell marker nestin, whereas only small subpopulations expressed lineage-restrictive markers such as the astrocyte marker GFAP (**Figure 2A**), the neuronal marker class III β-tubulin (**Figure 2B**), or the oligodendrocyte marker O4 (**Figure 2C**). When culture medium was replaced with oligo medium + GF and cells were cultured for 1 week, decreased nestin expression and increased A2B5 expression can be observed (**Figure 2D**). A2B5 is a neuroglial precursor marker. In comparison, 2 weeks after medium replacement, nestin expression was decreased even further and A2B5 expression alone increased (**Figure 2E**) as well as the expression of another oligodendrocyte marker, O4 (**Figure 2F**). Two days post growth factor withdrawal, O4 expression increased as well as the expression of GalC, a late oligodendrocyte marker (**Figure 2G**). Six days post growth factor withdrawal, the co-expression of O4 and GalC increased (**Figure 2H**) and is comparable to the co-expression of GalC and MBP (**Figure 2I**). The multi-epitope immunostaining reveals that more than half of the cells in culture are expressing MBP (**Figure 2J**). Further evidence of PDO differentiation was characterized, using an immunofluorescence assay, by the temporal increase of MBP expression in these cells after growth factor withdrawal (**Figure 3A-C**). In summary, human fetal neural progenitor cells are able to proliferate *in vitro* while maintaining the capacity to differentiate into 3 major brain cell types (**Figure 4**).

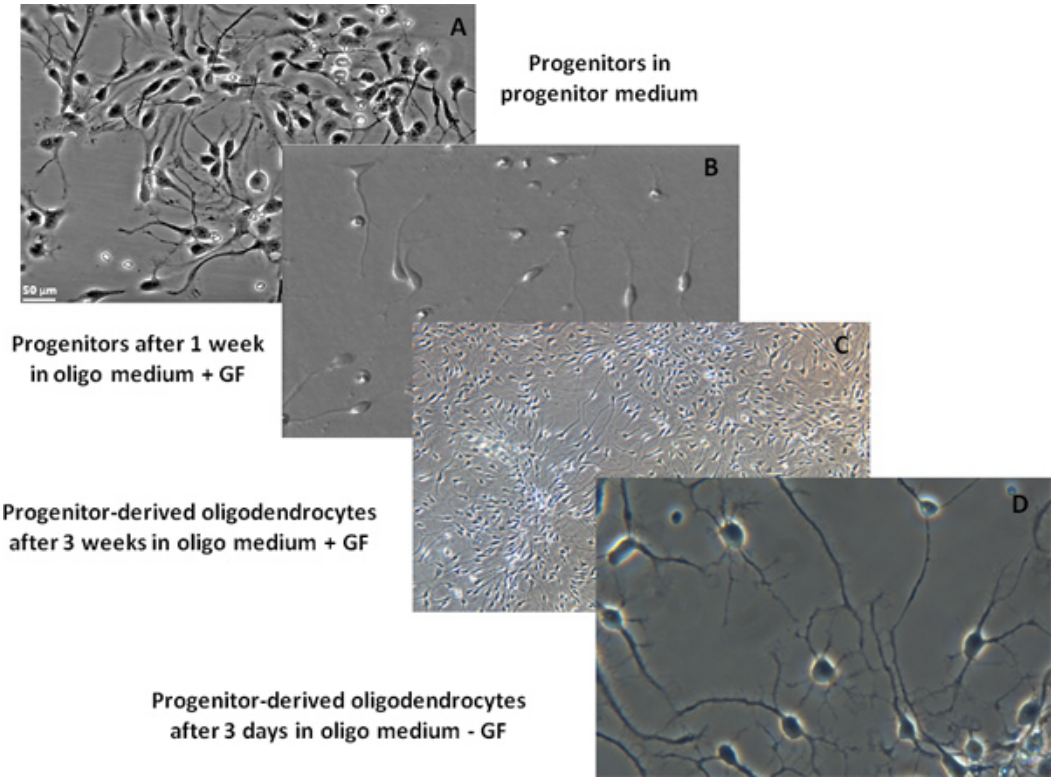

Figure 1. Phase contrast microscopy of **(A)** neural progenitor cells cultured in progenitor medium; **(B)** neural progenitor cells grown in oligo medium + GF for a week exhibit an altered narrow, bipolar morphology; **(C)** differentiated progenitor-derived oligodendrocytes grown for 3 days in oligo medium after growth factor withdrawal exhibit oligodendrocyte morphology characterized by multiple processes. 20x magnification.

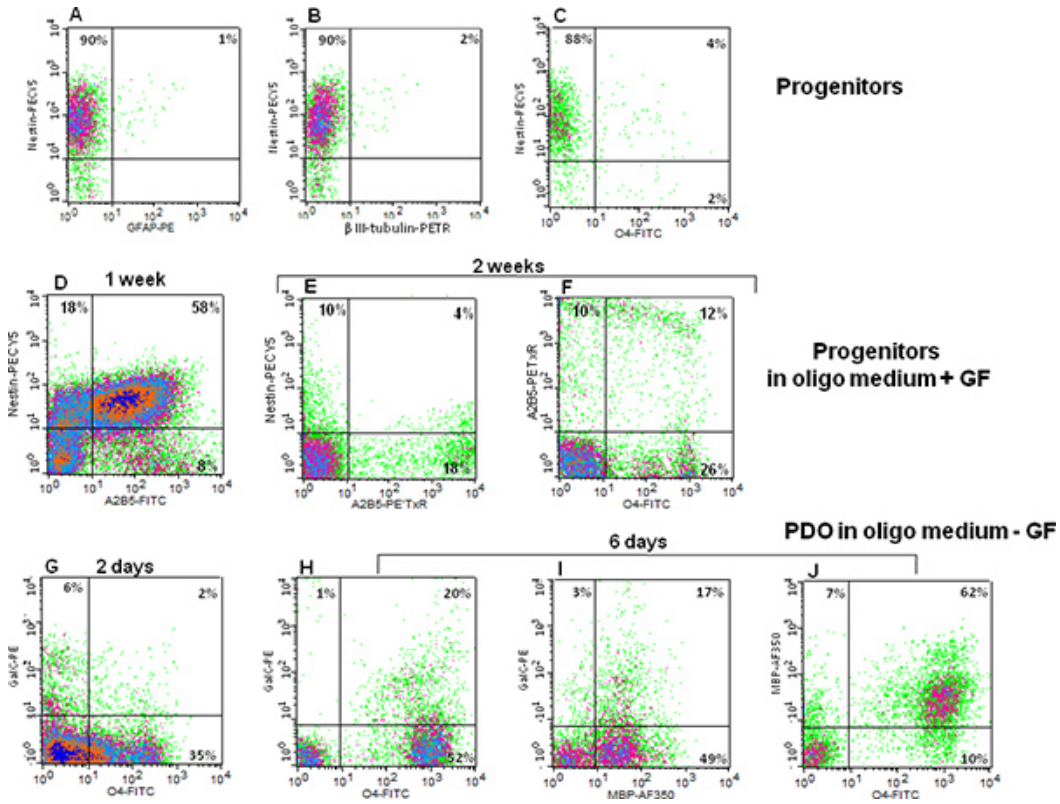

Figure 2. Flow cytometry analysis of neural progenitor cells co-expressing the precursor-cell marker Nestin (all vertical axes) with: **(A)** the astrocytic marker GFAP; **(B)** the neuronal marker β III tubulin; and **(C)** the oligodendrocytic marker O4; **(D)** neural progenitor cells grown for a week in oligo media + GF co-express nestin and A2B5; (**E)** nestin and A2B5 co-expression after two weeks of neural progenitors growth in oligo medium + GF **(F)** A2B5 and O4 co-expression after two weeks of neural progenitors growth in oligo medium+ GF; **(G)** two days after growth factor withdrawal, distinct oligodendrocyte markers of differentiation, O4 and GalC, are expressed. Six days after growth factor withdrawal **(H)** an increased proportion of cells are double-positive for O4 and GalC; **(I)** double-positive for GalC and MPB; and **(J)** double-positive for O4 and MBP. A-J are representative of four independent experiments. Click here to view larger figure.

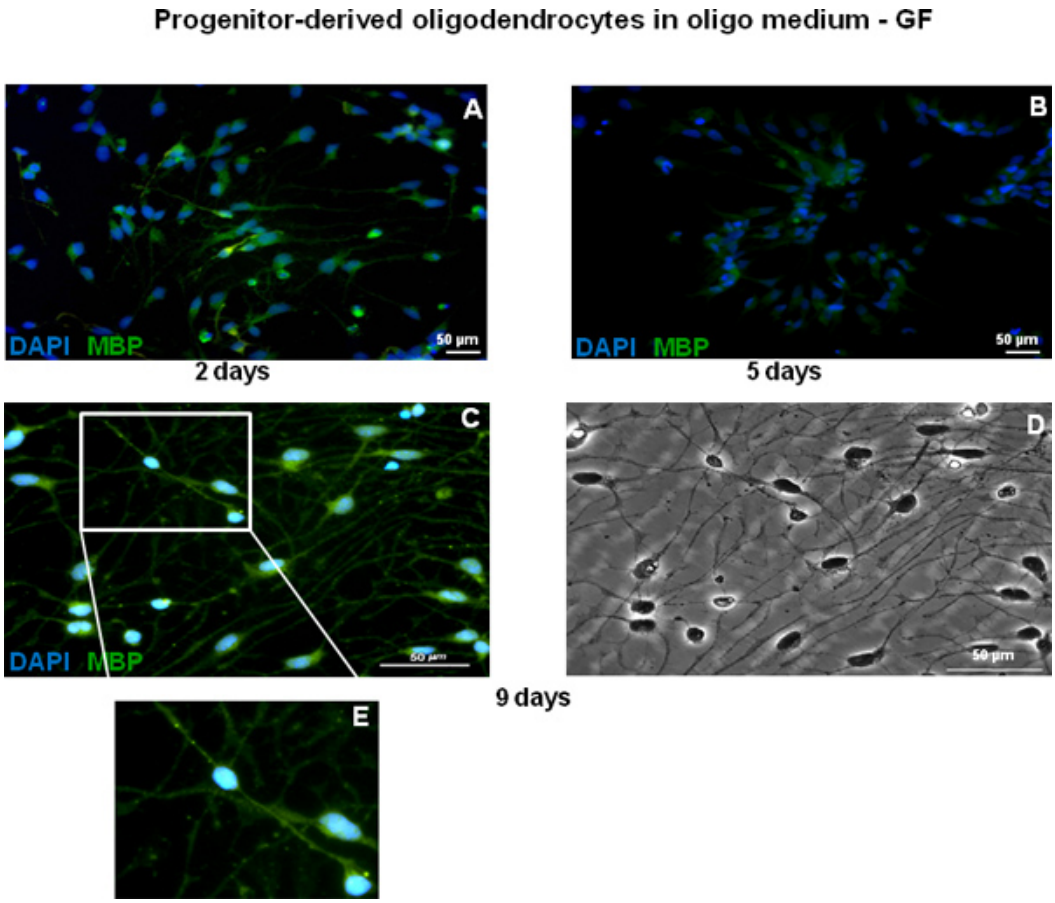

Figure 3. Indirect immunofluorescence staining of progenitor-derived oligodendrocytes for MBP **(A)** 2 days, **(B)** 5 days, and **(C, D)** 9 days after growth factor withdrawal. (**D)** Represents the phase image of the 9 days culture in oligo medium - GF and **(E)** is an enlargement of the white box of the same image. **(A, B)** 20x magnification; **(C, D)** 32x magnification.

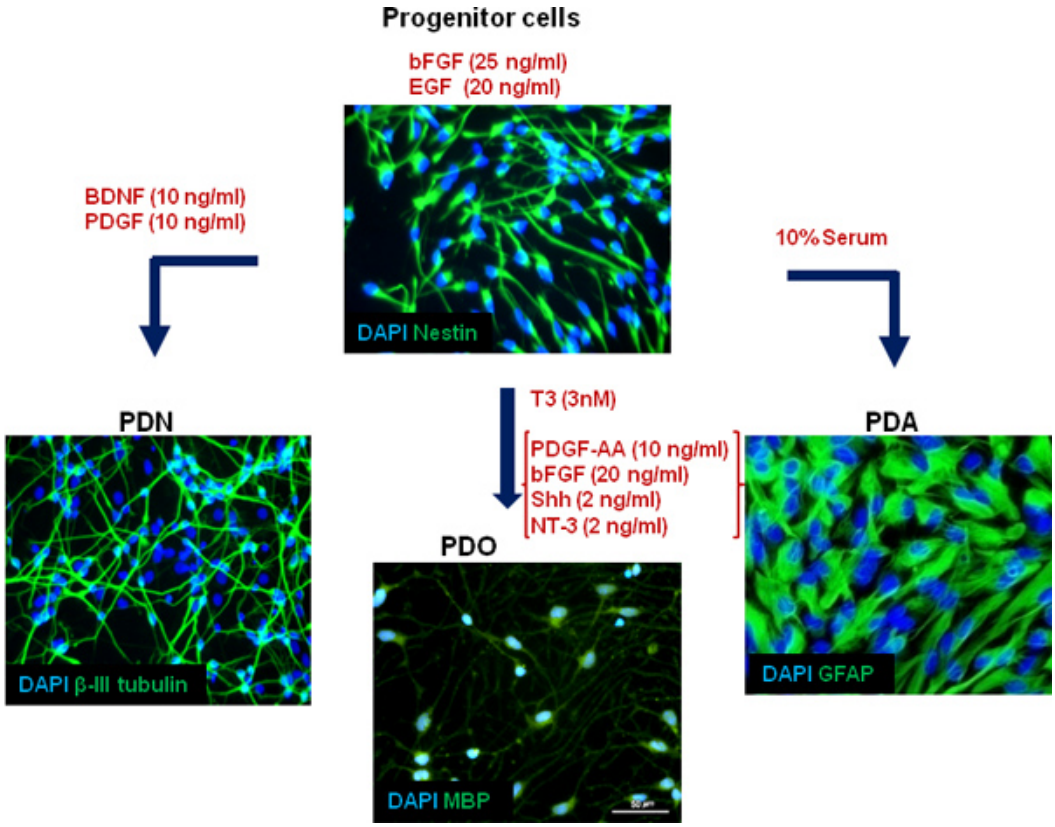

Figure 4. Schematic representation of our cell culture model. Primary, human fetal brain-derived, multipotential neural progenitor cells proliferate *in vitro* while maintaining their plasticity. Using different culture conditions, neural progenitor cells can differentiate into neurons (PDN), astrocytes (PDA) and oligodendrocyte (PDO), as shown by specific differentiation markers.

Discussion

This protocol describes how to derive fetal oligodendrocytes from primary human neural progenitor cells and characterize their phenotype using both flow cytometry and immunofluorescence staining. The expansion and growth of neural progenitors from fetal CNS has been very well described[1-4]. However, obtaining sufficient human oligodendrocytes for *in vitro* experimentation remains difficult, even though it is possible to identify and isolate glial precursors from adult human white matter[5-13]. There have been different attempts in the development of various culture conditions to direct differentiation of fetal neural progenitors into myelin producing oligodendrocytes[14-21]. This protocol further describes nestin positive neural progenitor cells expressing the O4 marker (**Figure 2**) when grown for 3 weeks in a serum-free medium supplemented with select growth factors (PDGF-AA, bFGF, Shh, and NT-3), that are essential for the proliferation and survival of oligodendrocyte precursors[22-24] . Removal of these growth factors in the O4+ cells resulted in further differentiation and expression of myelin components (galactocerebroside and MB). In addition, the expression of oligodendrocyte differentiation markers coincides with morphological changes from cells that at first appeared narrow and bipolar (**Figure 1B**) to cells that become multipolar with well-developed processes (**Figure 1C**). The removal of growth factors to further allow the final steps of differentiation was based on studies done both in mouse and human[15,25]. The presence of triiodothyronine (T3) is important for the survival and differentiation of oligodendrocytes[26] while we observed that the removal of the four growth factors was necessary to the expression of final markers of differentiation. This was compared with cells kept in oligo medium + GF as a control.

We are not the first to describe the differentiation of multipotent neural progenitor cells to oligodendrocyte cells in response to signals such as Shh and bFGF[24]. Previous reports showed that cortical oligodendrogenesis begins around 10 weeks gestational age in humans[24] with the capacity of human fetal oligodendrocytes to myelinate increasing proportionally with gestational age[22]. Differentiation of galactocerebroside+ and $O4^+$ oligodendrocyte precursor cells from neural progenitor cells has been reported using second trimester fetal brain[21,27]. However, these cells do not proliferate in the absence of support cells including astrocytes and neurons, and are lost quickly over time in culture[21]. Our system supports the formation of $GalC^+$ and MBP^+ cells from human fetal culture from 8 weeks gestational age. Furthermore we described that the differentiated oligodendrocytes could be cultured *in vitro* without the need of supporting cells as astrocytes or neurons, although the co-cultivation with neurons could lengthen the survival of the differentiated oligodendrocytes. One more benefit of this protocol is the possibility to cultivate a large number of cells expressing the $O4^+$ marker that can be grown and passaged in culture for weeks while maintaining their phenotype. At that moment those $O4^+$ cells can be pushed further in the final step of differentiation when the growth factors are removed from the medium. The protocol outlined in this paper addresses the need for oligodendrocyte lineages suitable for *in vitro* experimentation. Moreover, we believe that the identification of specific factors that induce the differentiation cascade of neural progenitors towards progenitor-derived oligodendrocyte

is important for understanding the cellular and molecular mechanisms of developmental transitions. It may also serve as an important tool for studying demyelinating disorders and virus-host cell interactions relate to the pathogenesis of human neurotropic viruses such as JCV.

Disclosures

No conflicts of interest declared.

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