Research Paper

Induction of rat neural stem cells into oligodendrocyte precursor cells

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Abstract: We have previously established a culture method to isolate and cultivate neural stem cells (NSCs) derived from the rat embryonic brain and spinal cord. In the present study, we demonstrate that the spinal cord-derived NSCs can be induced to differentiate into oligodendrocyte precursor cells (OPCs) with a combined treatment composed of (1) conditioned medium collected from B104 neuroblastoma cells (B104CM) and (2) basic fibroblast growth factor (bFGF, 10 ng/ml). After induction, over 95% of the cells displayed bipolar or tri-polar morphology and expressed A2B5 and platelet derived growth factor receptor-α (PDGFR-α), markers that are specific for OPCs. Among PDGFR-α positive OPCs, only a few cells expressed glia fibrillary acidic protein (GFAP) and none expressed β-tubulin Ⅲ. In the presence of B104CM and bFGF, OPCs proliferated rapidly, formed spheres, expanded for multiple passages, and maintained their phenotypic properties. Upon withdrawal of B104CM and bFGF, these cells differentiated into either O4/GlaC-positive oligodendrocytes (OLs) or GFAP- and A2B5-positive type-2 astrocytes. Our results indicate that NSCs can be induced to differentiate into OPCs that possess properties of self-renewal and differentiation into oligodendrocytes and type-2 astrocytes, a property similar to that of O-2A progenitor cells. The OPCs can be maintained in an undifferentiated state over multiple divisions as long as both B104CM and bFGF are present in the medium. Thus, large quantity of OPCs can be obtained through this method for potential therapeutical interventions for various neurological degenerative diseases.

Key words: neural stem cells; oligodendrocyte precusor cells; B104 neuroblastoma cells; basic fibroblast growth factor

神经干细胞向少突胶质前体细胞的定向分化诱导

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摘 要: 本研究采用神经胶质瘤细胞株(B104 neuroblatoma cells, B104 cells)培养上清(B104CM)和碱性成纤维细胞生长因子(basic fibroblast growth factor, bFGF), 将冷冻复苏的大鼠胚胎脊髓神经干细胞(neural stem cells, NSCs)定向诱导为少突胶质前体细胞 (oligodendrocyte precursor cells, OPCs)。形态学和免疫组化的结果显示, 诱导后95% 以上的细胞具有双极或多极突起的典型OPCs 形态, 并表达 A2B5 和血小板源生长因子受体 -α (platelet derived growth factor receptor-α, PDGFR-α)等OPCs 标志, 所有 PDGFR-α 阳性的OPCs 均不表达β-Tublin Ⅲ, 其中仅少量细胞表达胶质原纤维酸性蛋白 (glia fibrillary acidic protein, GFAP)。在 B104CM 和 bFGF 共存的培养条件下, 悬浮培养的OPCs 可大量增殖形成少突胶质细胞球, 该细胞球可通过传代继续扩增, 且扩增的OPCs 仍能维持其特有的形态和自我增殖的特性。撤去 bFGF 和 B104CM 后, OPCs 能进一步分化为成熟的少突胶质细胞 (oligodendrocytes, OLs) 或Ⅱ型星形胶质细胞。实验表明, 诱导 NSCs 产生的OPCs 在形态、增殖以及分化格局等方面均与已报道的存在于胚胎脑区的 O-2A 前体细胞相类似。该培养系统可为实验性细胞移植的研究提供丰富的细胞来源。

关键词: 神经干细胞; 少突胶质前体细胞; B104 细胞; 碱性成纤维细胞生长因子
中图分类号: Q421; Q593[+].2

Oligodendrocytes (OLs) are best known as the myelin-forming cells of the central nervous system (CNS). They wrap axons of neuron to produce myelin. Myelination of axons allows the electrical insulation, facilitates conduction of electrical signals between neurons[1] and provides trophic support and protection for neurons and their axons[2]. Oligodendrocyte precursor cells (OPCs) are immature oligodendrocytes and can differentiate into myelin-forming cells of the CNS under certain conditions. Many studies have shown that OPCs could form new myelin around demyelinated axons after being transplanted into myelin-deficient animals [3-6] and promote regeneration of injured axons after being transplanted into spinal cord injured animals [7]. Thus, transplantation of OPCs shows promise as an attractive therapy for treatments of CNS injury or demyelination.

In general, OPCs can be isolated from the brain, spinal cord and optic nerve of embryonic or neonatal animals, purified by sequential immunopanning, and cultured in chemically defined medium *in vitro* [8]. However, this method is restricted in practice due to its complicated procedures and costs. Recently, Zhang *et al.* reported that OPCs could be induced from neural stem cells (NSCs) by the use of conditioned medium obtained from B104 neuroblastoma cells (B104 CM) [3, 7, 9-11], which provides an alternative approach to obtain OPCs. Since NSCs can be propagated ceaselessly *in vitro* and frozen conveniently, to generate OPCs from NSCs may be more advantageous than to harvest them from the brain tissue directly. In the present study, we established a culture method in which OPCs can be induced from NSCs derived from the rat embryonic spinal cord using both B104CM and bFGF. Under the influence of the combined treatment, the OPCs proliferated, passaged and differented into oligodendrocytes and type-2 astrocytes. The present work therefore has laid a foundation for further investigation of using these cells in the treatments of CNS degenerative or demylinating diseases.

1 MATERIALS AND METHODS

1.1 *Cultivation of NSCs derived from the embryonic rat spinal cord*

All embryonic rats were obtained from female pregnant rats (Wistar, E16). Isolation, cultivation and cryopreservation of NSCs were performed according to our previous report[12, 14]. The culture medium of NSCs (referred as NSC-M) was composed of DMEM/F12 (1:1), N2 (1%), heparin (2 μg/ml), glutamine (2 mmol/L), and freshly added bFGF and EGF (20 ng/ml).

1.2 *Preparation of B104 CM*

B104 neuroblastoma cells were a generous gift from Dr. Ian Duncan (University of Wisconsin) and B104CM was prepared according to Louis's method [13].

1.3 *Induction, purification and passage of OPCs*

The OPC-growth medium (referred as OPC-M) comprised NSC-M (without bFGF/ EGF) and B104CM in the proportion 7:3 and was supplemented with bovine serum albumin (BSA, 0.1%), biotin (10 ng/ml) and bFGF (10 ng/ml). To generate OPCs from neurospheres, we gradually changed the bFGF/EGF-containing NSCs-M to B104CM-containing OPCs-M by replacing one third of the former medium with the latter every 3 d. With time, the majority of cells in the neurospheres migrated out and attached to the bottom of the flask. These attached cells showed morphological characteristics of OPCs. Simultaneously, some necrotic spheres generated and floated within the medium. At this time, the old medium was replaced by fresh OPC-M to eliminate necrotic spheres and fragmented cells. Cells were cultured for another 5~7 d and new spheres were generated, which were referred as oligospheres. For passage, cells suspension was transferred to a 15-ml tube and was centrifuged for 8 min at 800 r/min. The supernatant was discarded. Two hundred microliters of fresh OPC-M was used to resuspend the OPCs into single cell suspension with gentle trituration. Finally, cells were plated at a density of 2×10^4 cells/cm² in OPC-M.

1.4 *Immunofluorescence staining.*

For fluorescent labeling experiments cells were grown on poly *D*-lysine-coated (PDL, 200 μg/ml) glass cover slips in 35 mm dishes at a density of 3×10^4 cells/coverslip (1.13 cm²) and cultured under different conditions for 3 or 5 d. After washing with PBS (0.01 mol/L, pH7.4), cells were fixed with 4% paraformaldehyde (PFA) for 20 min. For intracellular staining , PFA-fixed cells were pre-treated with 0.3% Triton X-100 containing 10% normal goat serum (NGS) for 60 min and were incubated with the first antibodies overnight at 4 ℃. The antibodies were used at the following dilutions: anti-nestin (mouse IgG) 1:800; anti-β Ⅲ -tubulin (mouse IgG) 1:200; anti-GFAP (mouse IgG) 1:200; anti-PDGFRα (rabbit IgG) 1:100; anti-A2B5 (mouse IgM) 1:100; anti-O4 (mouse IgM) 1:100; and anti-GalC (mouse IgM) 1:100. After washing with PBS, cells were incubated with FITC-conjugated goat anti-mouse IgG (1:120) or IgM (1:200) and Rho-conjugated goat-rabbit IgG (1:80) for 60 min at 37 ℃, respectively. The cells were washed with PBS and mounted with Gel/Mount aqueous mounting media containing Hoechst 33342 (1 μg/ml), a

fluorescent nuclear dye, and observed using an Olympus BX60 microscope.

2 RESULTS

2.1 Cultivation and identification of NSCs

In the presence of EGF and bFGF, single NSCs proliferated and formed free-floating neurospheres. These cells were immunopositive for nestin, an intermediate filament protein mainly expressed by stem or precursor cells [15]. When the spheres were triturated into single cells and plated onto PDL-coated coverslips in the NSC-M containing 1% fetal bovine serum (FBS) in the absence of EGF/bFGF, they differentiated into a mixture of astrocytes, oligodendrocytes and neurons (data not shown). Through multiple passages or cyropreservation, the NSCs remained capac-
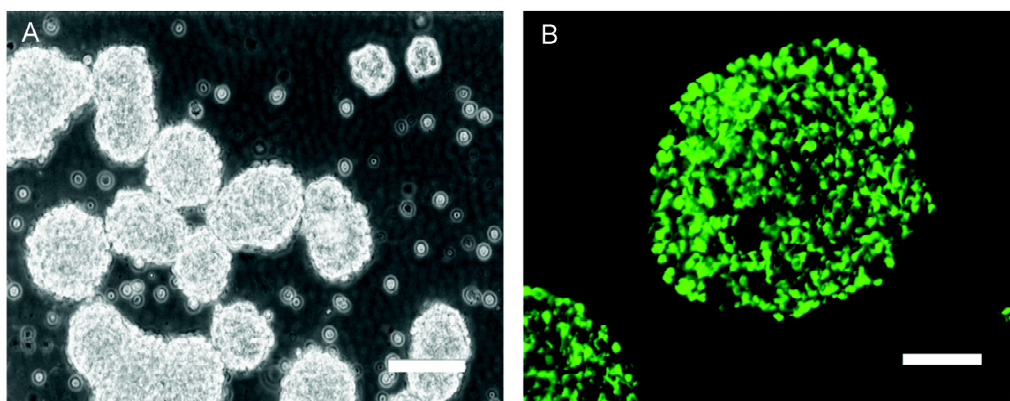

Fig. 1. NSCs formed neurospheres and were nestin-positive. *A*: Phase-contrast photograph shows numerous neurospheres grown in serum-free medium supplemented with bFGF and EGF for 5 d at passage 3. Scale bar, 100 μm. *B*: Cells in the neurosphere are immuno-positive for nestin. Scale bar, 50 μm.
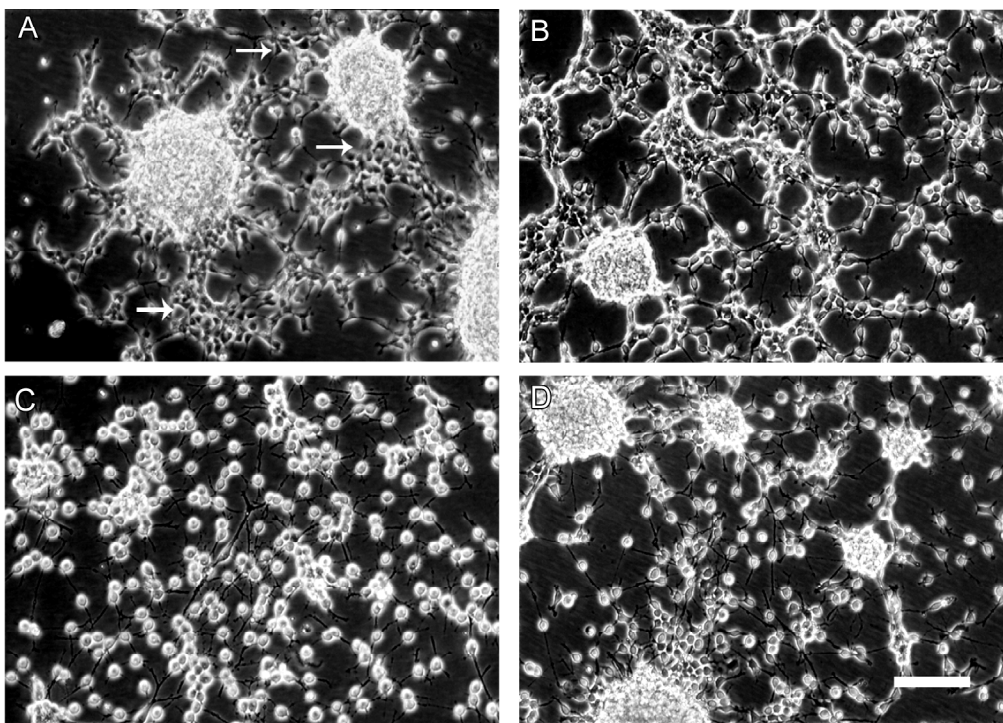

Fig. 2. Generation of OPCs from NSCs and the formation of oligospheres. *A*: In the presence of 30% B104CM and bFGF for 2 d, some cells migrated from the neurospheres that attached to the flasks displayed morphology of astrocytes (arrows). *B*: At 7~10 d, increased numbers of migrating cells showed bipolar or tri-polar processes. *C*: Adhering cells with bipolar or tri-polar processes remained in the bottom of flasks after the removal of degenerated spheres. *D*: These cells proliferated, formed oligospheres and could be passaged for multiple times. Scale bar, 100 μm.
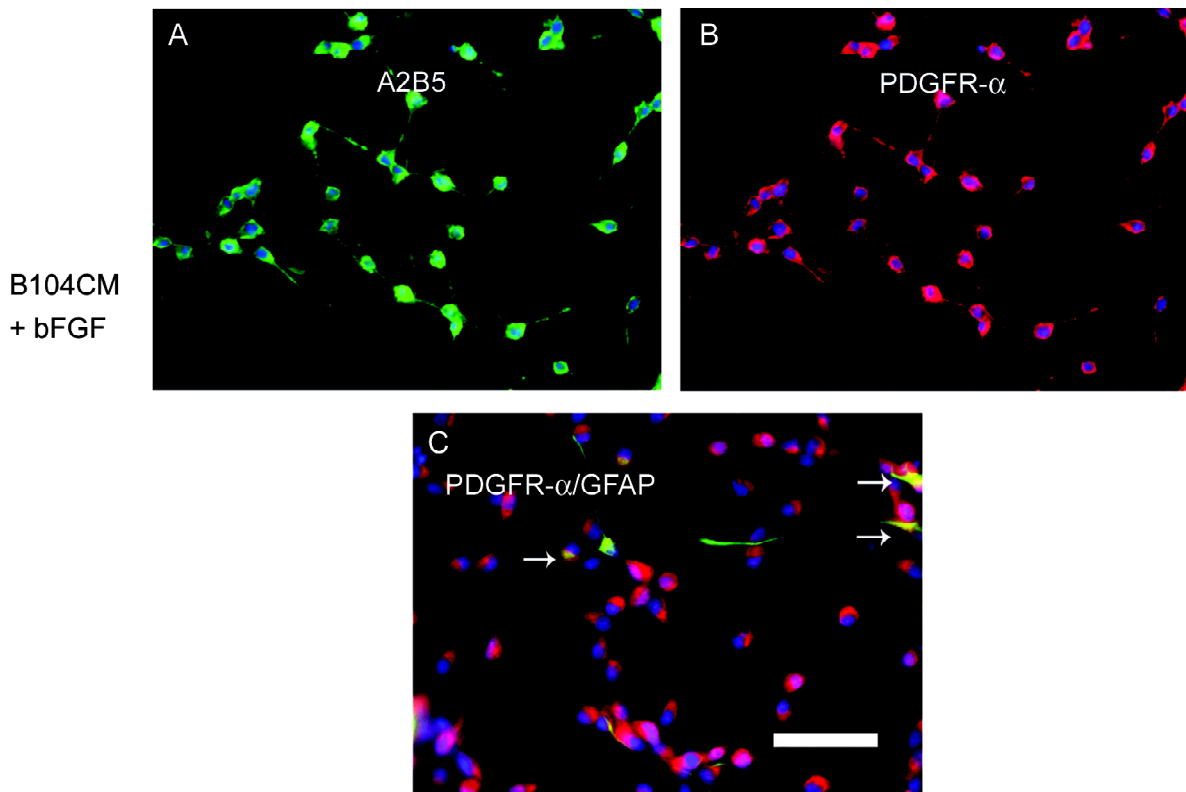
Fig. 3. Characterization of OPCs. *A*, *B*: OPCs, derived from NPCs, expressed both A2B5 (*A*, green) and PDGFR-α (*B*, red). *C*: Among PDGFR-α (red)-positive OPCs, only a few cells expressed GFAP (green). All cell nuclei were labeled with Hoechst 33342 (blue). Scale bar, 25 μm.
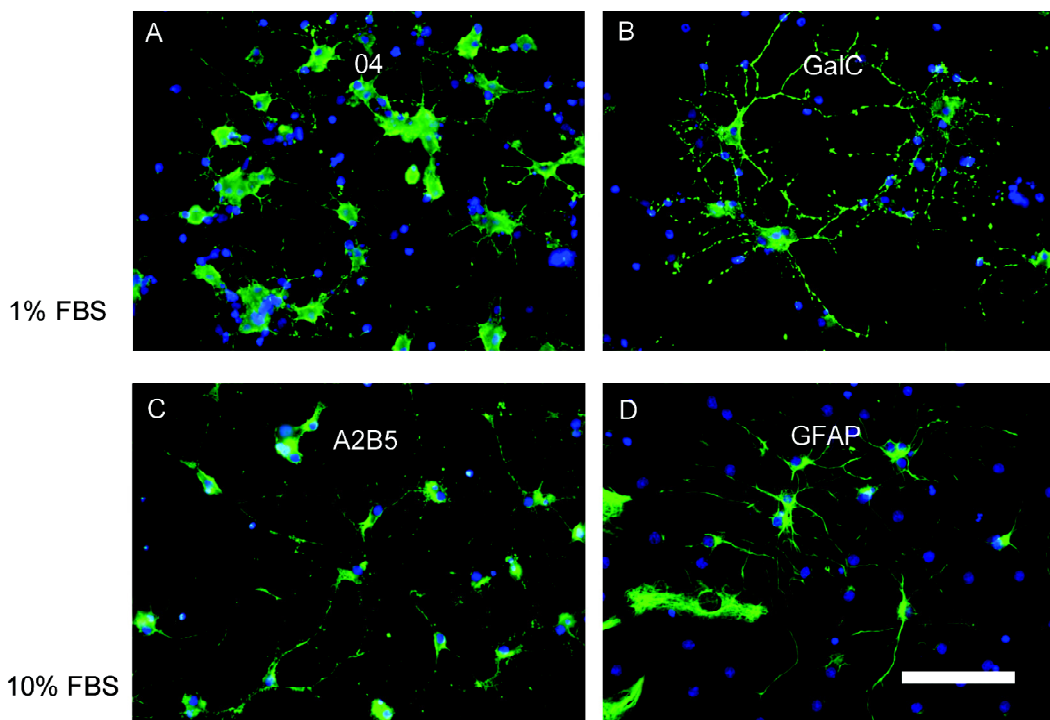

Fig. 4. Differentiation of OPCs. *A*, *B*: In the presence of 1% FBS and absence of growth factors , many OPCs expressed O4 at 3 DIV or GalC at 5 DIV. *C*, *D*: When cultured in the presence of 10% FBS, the majority of cells showed flattened cell bodies and star-shaped processes and were positive for both A2B5 and GFAP, the characteristic marks of type-2 astrocytes. All cells nuclei were labeled with Hoechst 33342 (blue). All cell specific markers were in green (fluorescein). Scale bar, 25 μm.

ity to survive, proliferate and differentiate into neurons and glial cells *in vitro*. Fig. 1 shows that NSCs at passage 3 (P3) formed neurospheres and were positive for nestin at 5 d *in vitro* (DIV).

2.2 Morphological characteristics of OPCs differentiated from NSCs

After the NSC-M medium was gradually replaced by the OPC-M medium, the floating neurospheres sank to the bottom of culture flasks and many cells migrated out from the spheres. The cells that migrated out at an early phase contained larger cell bodies with short processes, which are similar to astrocytic morphology (Fig. 2*A*). At about 7~10 d later, the cells migrated from the neurospheres displayed small, ellipse cell bodies and symmetrical bipolar or tri-polar processes, typical of OPCs (Fig. 2*B*). After active cell migration, the remaining spheres gradually contained mainly dead cells and cell fragments, lost their 3-dementional morphology, and were lifted from the bottom of the flasks. These degenerating spheres were removed from the culture so that only cells that attached to the bottom of flask would be maintained. These cells displayed bipolar or tri-polar morphology, typical of OPCs (Fig. 2*C*). These cells further proliferated and formed new spheres in the OPC-M, which were now referred to as "oligospheres" (Fig. 2*D*).

2.3 OPCs characterization and differentiation

When oligospheres were triturated into single cells and plated onto PDL-coated coverslips in the OPC-M, all cells displayed bipolar or tri-polar morphology, expressed both A2B5 (Fig. 3*A*) and PDGFR-α (Fig. 3*B*), but were negative for the neuronal marker β-tubulin III. Moreover, PDGFR-α and GFAP double-staining (Fig. 3*C*) showed that only a few OPCs expressed GFAP suggesting that the purity of OPCs induced by this method was high ($\geq$ 95%). To assess their differentiation potential, the OPCs were cultured in the presence of 1% or 10% FBS. After 3~5 d, these cells differentiated into O4/GalC-positive oligodendrocytes (Fig. 4*A*, *B*) or A2B5- (Fig. 4*C*) and GFAP-positive (Fig. 4*D*) type-2 astrocytes, respectively. These results suggest that the characteristics of these OPCs, induced from the NSCs, were similar to those of O-2A progenitors in the embryonic brain as were reported previously [6, 13, 16, 17].

2.4 Proliferation property of OPCs

Oligospheres were triturated into single cells and passaged in OPC-M by plating 5×10^5 cells into a 25 cm² flask. After 5~7 d, new oligospheres formed and passaged again. Through this approach, large quantity of OPCs was obtained. As shown in Fig. 5, OPCs derived from 4 differ-
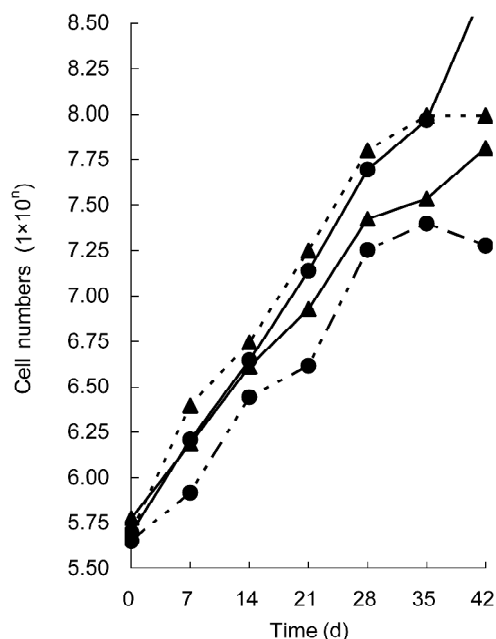

Fig. 5. Growth rates of OPCs *in vitro*. The OPCs were generated from 4 individuals and passaged for 7 continuous passages (up to 49 d). Each data point represents the total number of cells obtained at the date of passage.

ent individuals were expanded for 7 passages. The OPCs from all individuals displayed consistent and rapid proliferation. However, the rate of proliferation decreased with increased times of passage. From P5 (35 d) or so, proliferation rates of OPCs began to decline and the difference in proliferation rate between individuals were increased.

3 DISCUSSION

In the present study, we demonstrated that NSCs derived from the embryonic rat spinal cord could be induced to differentiate into OPCs in the presence of B104CM and bFGF. More importantly, large quantities of highly purified OPCs (>95%) can be generated through this modified approach and can be used as a reliable source for cell therapy.

Although B104CM has been reported to play an important role in inducing OPC differentiation from brain-derived NSCs [3,7,9-11], the procedure employed in the present study has expanded this approach in several ways. First, we demonstrate that OPCs can be induced from the NSCs originated from the embryonic spinal cord. This observation can be practically important since OPCs obtained from

different regions of the CNS may be regional specific. Our long-term goal is to use OPCs as a source of cell therapy to promote regeneration and remyelination of damaged axons following spinal cord injury. Thus, it is important to characterize these cells and compare them with those obtained from other regions to determine the feasibility of using a particular cell population for transplantation.

Second, we used both B104CM and bFGF in our culture system, which greatly enhanced the survival and proliferation of the OPCs, compared with B104CM alone. This suggests that bFGF is critical for the survival and proliferation of OPCs. Although bFGF is required for the maintenance and, more importantly, passage of OPCs in the current study, mechanism underlying its action remains to be elucidated.

Lastly, several methodological improvements were made to purify OPCs in the present study. For example, we discarded degenerating neurospheres after the migration and attachment of OPCs to the bottom of flasks. This procedure greatly enhanced the purity and vitality of OPCs. OPCs grew and proliferated much better after degenerating spheres and fragments of cells were removed. These spheres, if replated, grew much slower and showed poor vitality and purity compared with those attached to the bottom of the flask. Why the OPCs attached to the bottom grew better than those derived from degenerating spheres? We speculated two possibilities. First, cells attached to the bottom of the flask were more active than those existing within the spheres. Alternatively, differentiated astrocytes, although small in number, may provide growth factors [18] for the survival and proliferation of OPCs.

In the present study, neurospheres were used as a cell source for generating OPCs. A potential pitfall of this approach is that the accessibility of growth factors to the cells inside or outside of spheres may be different, which may, in turn, result in asynchronized differentiation of OPCs. Although this problem can be avoided by removing degenerating spheres, OPCs with strong proliferation capabilities may also be removed with the spheres. We also found that some OPCs died through apoptosis after several passages. This may be a reason accounting for the decline in OPC proliferation at later passages. Asakura *et al.* reported that B104 cells also secreted TGF-β[19]. Whether apoptosis-inducing factors such as TGF-β in the B104CM play a role in OPC apoptosis remains to be addressed. Blocking these factors may improve the survival and proliferation of isolated OPCs, which could be an intriguing topic of future studies.

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