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The Cell Survival and Differentiation after Transplantation, Which Harvest from Adult Rat Brain by High-speed Centrifugation Method

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Objective : Many recent reports have shown that the mature mammalian brain harbors multipotent stem cells, rendering the brain capable of generating new neurons and glia throughout life. Harvested stem cells from an adult rat are transplanted in order to evaluate the cell survival and differentiation.

Methods : Using a percoll gradient with a high speed centrifugation method, we isolate neural stem/progenitor cells were isolated from the subventricular zone(SVZ) of a syngeneic adult Fisher 344 rats brain. For 14days expansion, the cultured cells comprised of a heterogeneous population with the majority of cells expressing nestin and/or GFAP. After expanding the SVZ cells in the presence of basic fibroblast growth factor-2, and transplanting then into the hippocampus of normal rats, the survival and differentiation of those cells were examined. For transplantation, the cultured cells were labeled with BrdU two days prior to use. In order to test their survival, the cells were transplanted into the dorsal hippocampus of normal adult Fisher 344 rats.

Results : The preliminary data showed that at 7days after transplantation, BrdU+ transplanted cells were observed around the injection deposition sites. Immuno-fluorescent microscopy revealed that the cells co-expressed BrdU+ and neuronal marker β-tubulin III.

Conclusion : The data demonstrate that the in vitro expanded SVZ cells can survive in a heterotypic environment and develop a neuronal phenotype in the neurogenic region. However more research will be needed to examine the longer survival time points and quantifying the differentiation in the transplanted cells in an injured brain environment.

KEY WORDS : Neural stem cell · Transplantation · Percoll solution · High-speed centrifugation · Subventricular zone · Hippocampus.

Introduction

Since, Cajal[29] pronounced that "In adult centers, the nerve paths are something fixed, and immutable : everything may die, nothing may be regenerated" the neuron was taken for granted to be in the G0 phase in its mitotic activity. Despite the occasional conflicting reports, this view remained the central dogma of developmental neurobiology for decades[12,26,31].

However, in the last two decades, the development of new techniques has resulted in an explosion of new research showing that neurogensis, the birth of new neurons, normally occurs in specific regions of the adult mammalian brain, and that there are significant numbers of multipotent neural precursors in many parts of the adult mammalian brain. Altman[9,29] was the first to use techniques sufficiently sensitive enough to detect the ongoing cell division that occurs in adult brain. Using tritiated thymidine as a mitotic label, he showed that neurogenesis constitutively occurred in the hippocampus and olfactory bulb of the adult mammalian brain. These results were later replicated using tritiated thymidine labeling followed by electron microscopy[12]. The field of adult neurogenesis was rekindled in 1992, when Reynolds and Weiss[9] showed that precursor cells isolated from the forebrain can differentiate into neurons in vitro. In addition, precursor cells isolated from

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the adult mouse ventricular zone and forebrain have the capacity for in vitro neurogenesis when stimulated with an epidermal growth factor or the basic fibroblast growth factor (bFGF-2), respectively. Beta FGF-2 is a potent mitogen for fetal cells isolated from different areas of the brain[5].

Density gradient media have been frequently used to fractionate cells on the basis of the buoyant density[17]. Mitotically-active neural progenitors are small, dense cells with a low nucleus to cytoplasm ration. In accordance with this, they exhibit low buoyancy in density gradients[4]. Palmer et al[14]. reported that neural progenitors have a relatively high-buoyancy density of 1.065~1.075gm/ml. Under the calibrated marker beads, the neural stem/progenitor cells were separate using the percoll solution.

Stem-like cells typically formed a discrete band at the bottom of the gradient between the red and blue marker beads. The harvested cells were cultured with the bFGF-2 mitogen stimulation for 2weeks and the cell characteristics were evaluated with the specific protein markers[17]. These cells were then transplanted into hippocampus of the normal adult Fisher 344 rats and the survival and differentiation of the transplanted cells were evaluated.

Materials and Methods

Cell suspension

The rats (170~190g, Fisher 344 males or females) were deeply anesthetized with isofluran inhalation (5 Mac) for 10minutes. The animals were decapitated, and the whole brains were removed. The harvested brains were transported in a cold phosphate buffer solution. Two coronal cuts were made in the areas between the rhinal fissure and the hippocampus. The resulting tissue chunk was laid on its posterior surface, and two parasagittal cuts were made just lateral to the lateral ventricles, and one horizontal cut was made at approximately the level of the corpus callosum. The neurospheres were made by dissociating the central, rectangular piece of tissue containing the lateral ventricles[4,16,17].

The tissues were minced (1mm^3) with a knife and the tissues were digested in a solution of papain (2.5U/ml; Worthington, Freehold, NJ) dissolved in HBSS for 30minutes in 37° C. The cells and tissue fragments were passed serially through ascending gauge needles (16G, 18G, 20G, and 24G), and washed three times with DMEM containing 10% fetal bovine serum (FBS)(Hyclone, Logan, UT). The whole digested tissue was then suspended in DMEM-10% FBS, and filtered through a sterile 50um nylon mesh. The percoll solution was made by mixing nine parts of percoll (amersham Pharmacia Biotech, Uppsala, Sweden) to one part 10X PBS (Irvine Scientific, Santa Ana, CA). The cell suspension was then fractionated by high-speed centrifugation for 30min. 18C, at 20,000g, using with 20μg of blue and red marker beads. The cell fractions between the red and blue markers were harvested and washed free of Percoll by three or more rinses in DMEM-10% FBS[16,17].

Cell culture

The cells fractionated through the Percoll gradients were washed free of Percoll and plated onto Polyornithine/Laminin (Sigma, Saint Louis, MI)-coated multi-well dishes and flasks (Fisher Scientific, Houston, TX)[15]. For a 24hour acute culture, the cells were allowed to attach to the glass slides in DMEM/F-12 (1:1) containing 10% FBS(Gibco, Carlsbad, CA) and the culture medium consisting of DMEM/F-12 (1:1) susppplemented with N2 supplement (Life Technologies, Gaithersburg, MD) and 20ng/ml recombinant human FGF-2 prepared in Escherichia coli (San Diego, CA) subsequently changed every 48hours for 2weeks[16, 17].

Immunostaining

The culture media was removed after the cells has been cultured on polyornithine/laminin-cated glass chamber slides for 2weeks. The cells were fixed with 4% paraformaldehyde in PBS for 10min, rinsed three times (5min each wash) with PBS and treated as follows. The cells were first pre-incubated with PBS containing 5% pre-immune donkey serum (PBS-DS) for 30 minand then incubated with primary antibodies for the cell surface markers; Nestin (BD Biosciences, Palo Alto, CA), O4 (Chemicon, Temecula,CA) GalC (Sigma, Saint Louis, MI), GFAP (DAKO, Carpinteria, CA) and βtubulin-III (Covance, Berkeley, CA) in PBS-DS for overnight at 4° C. The cells were then washed three times, for 10minutes each with PBS. The cells were then incubated for 2hours with secondary donkey anti-mouse(=Cy-2, green color), and anti-rabbit (=Cy-3, red color) (Jackson Immuno Reseach, West Grove, PA) diluted 1:200 in PBST-DS.

The cells were then washed with PBS three times. The final wash contained 10ng/ml 4',6-diamindino-2-phenylindole (DAPI, Sigma) in PBS. This was used as a fluorescent counterstain for the cell nuclei. The slides were then coversliped in 100 mM Tris, pH 8.5, containing 25% glycerol, 10% polyvinyl alcohol (Air Products), 2.5% 1,4-diazobicyclo-[2.2.2]-octane (Sigma, Saint Louis, MI)[5,11,16]. The immunofluorescent positive cells were counted using an inverted fluorescent microscope.

Transplantation

For transplantation, the cultured cells were labeled with BrdU two days prior to use. The cells were harvested from the cell culture flask (25ml, flask), apply by applying trypsin-EDTA (3ml/each flask) for 5minutes and shaking the flask to detach

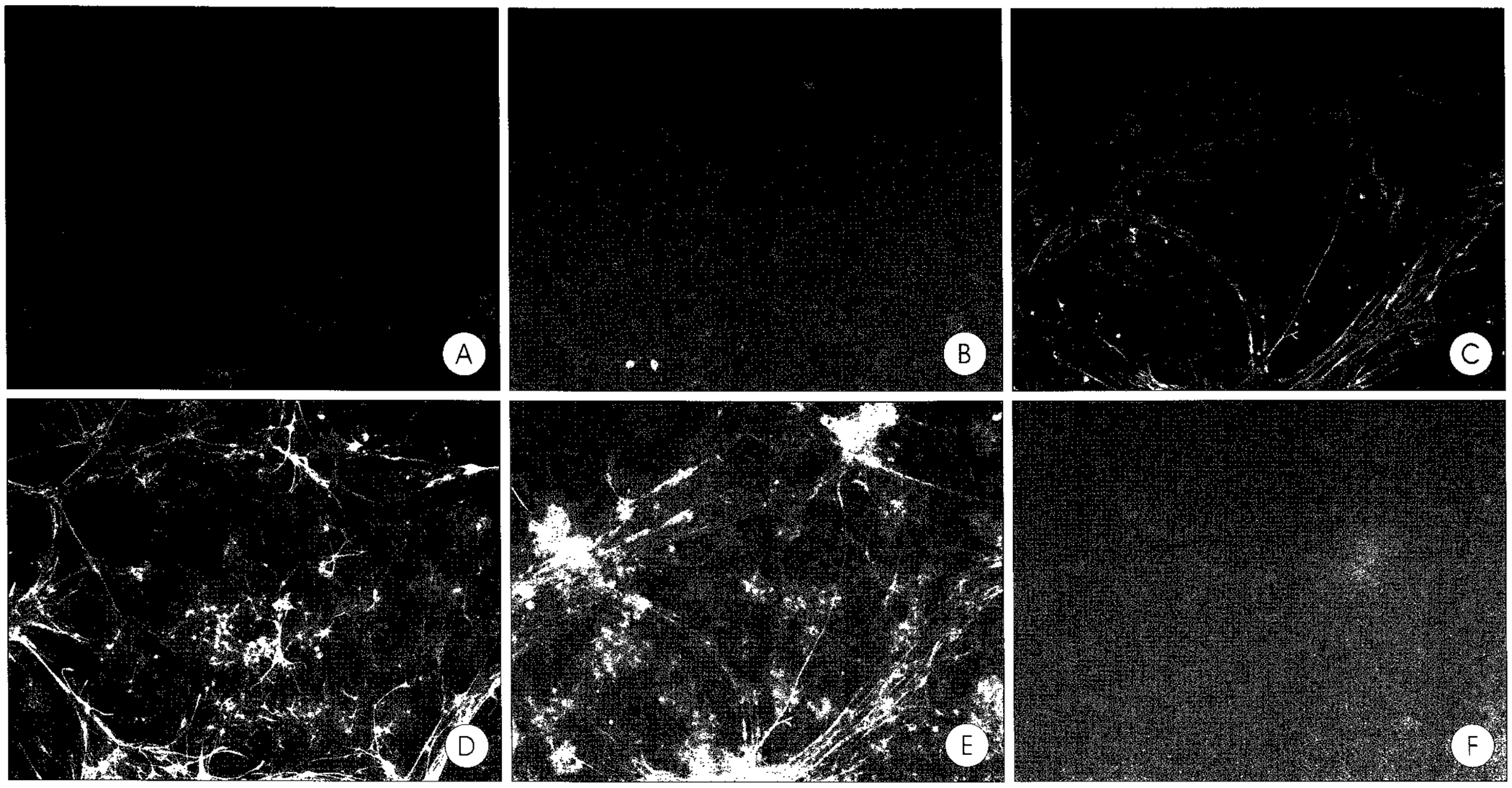

Fig. 1. Immunohistochemical staining after 2weeks cell culture(10X). Back ground staining with 4.6-diamindino-2-phenylindole (DAPI)(A). β-tubulin III staining (B). nestin (C). GFAP (D). O4 (E) and Gal-C (F).

the cells. Then apply the DMEM/F-12+ 10% Fetal Bovine Serum and centrifugation at 1,000rpm, 4° C for 10minutes for 3times to inactivate the typsin-EDTA[25]. The number of cells was counted using a Hemo-cytometer, and the cell concentration was made up to 20,000cell/μl.

The recipient rats were anesthesthetized with isofluran and the rat was placed on a small animal stereotactix frame, and 6μl of the cell suspension was injected into the dorsal hippocampus (AP = -0.4, Lat = 0.2 (left side), Depth = -0.3 from the Bregma) using a microinjection pump and a 10μl Hamilton syringe.

BrdU staining

For BrdU immunostaining, 6μm coronal sections were treated with 50% formamide, 280mM Nacl, 30mM sodium citrate at 65° C for 2hours and incubated in 2M HCl at 37° C for 30min. Sections were then rinsed in 0.1M boric acid (pH 8.5) at room temperature for 10minutes and then incubated in 1% H2O2 in TBS for 15minutes to block the endogenous peroxidase[2,33]. Using a BrdU staining kit (Zymed, San Francisco, CA) the sections were incubated with the mAb against BrdU overnight at 4° C.

The tissue specimens were then washed three times, for 10 minutes each with PBS, and then incubated for 2hours with secondary donkey anti-rabbit (=Cy-3, red color) (Jackson Immuno Reseach, West Grove, PA) diluted 1:200 in PBST-DS. The reaction product was detected using fluorescent microscopy.

Results

Characteristics of the cultured cell

Immuno-staining shows a relatively high positive incidence of the specific protein markers for the primitive cells. Positive incidence was 87.8% for nestin, 85.5% for GFAP, 82.6% for O4 and 91.5% for Gal-C (Fig. 1). However beta-tubulin III, which is a matured neuronal cell marker, was positive only in 1.6% of cells.

Cell survival and differentiation after transplantation

Preliminary data showed that BrdU+ transplanted cells were observed around the injection deposition sites, 7days after transplantation. Immuno-fluorescentmicroscopy revealed that the cells co-expressed BrdU+ and neuronal marker β-tubulin III (Fig. 2).

Discussion

There is a great deal of interest in stem cell therapy. Largescale sources of neural stem cells are essential for both basic research and novel approaches to treating neurological disorders[6,8,10,13,14,19]. Recently many reports emphasized that stem cells exist in the adult brain as well as in a developing embryo[30]. Many of neural stem cells resources are the basis of the cell lines that provide valuable research tools for general molecular and cellular neuroscience. Furthermore neural stem cells are seen as having significant potential in treating a wi-

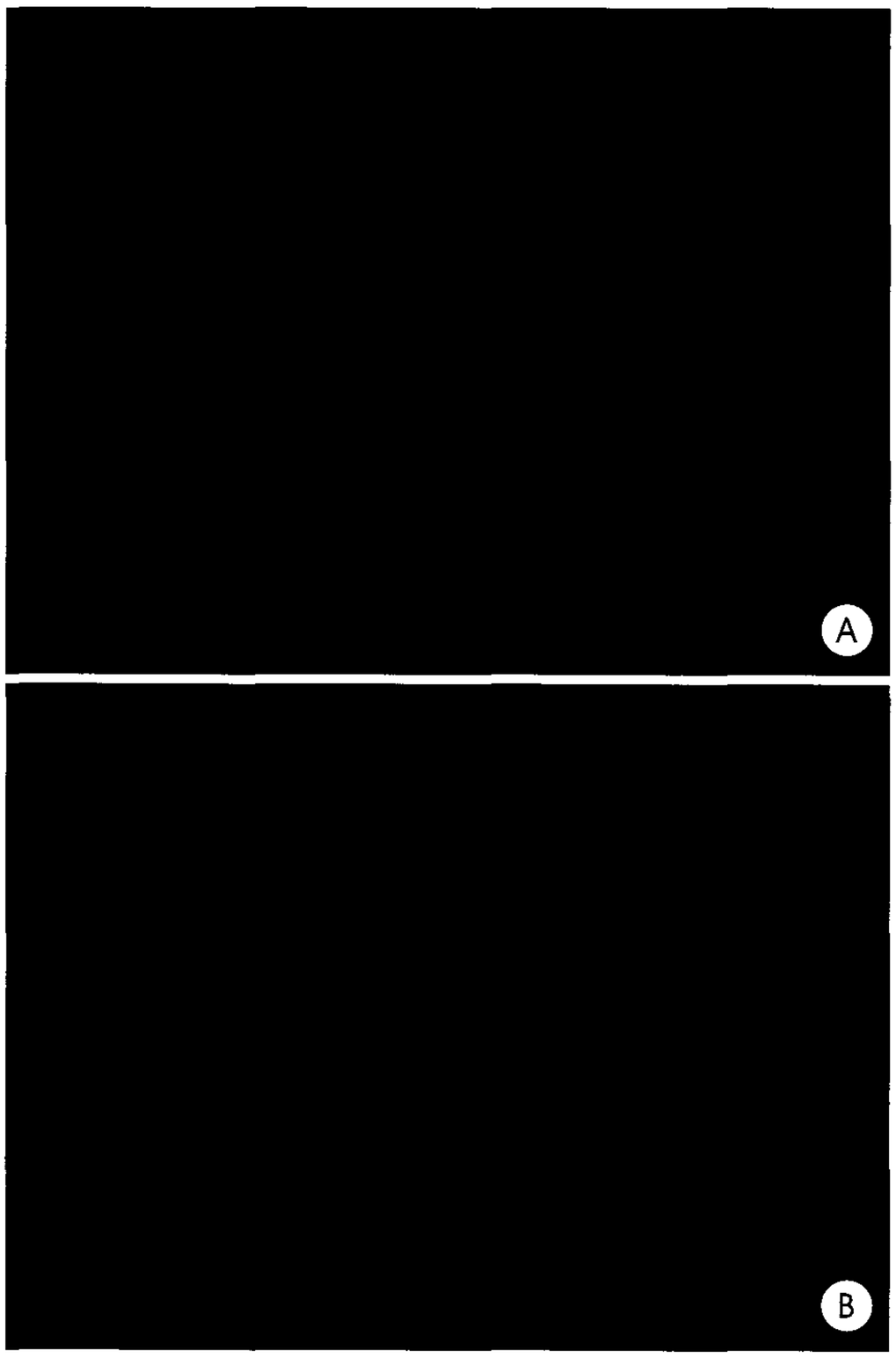

Fig. 2. Immunohistochemical staining after transplantation 7days (20X). BrdU staining (A) and β-tubulin III staining (B).

de range of clinical conditions involving the loss of neural cells[7,27,29,32]. This study, authors isolated adult neural stem cells through the high-speed centrifugation using the percoll gradient method, and suggest several possibilities regarding the presence of multipotent progenitors in the adult brain and provide a direction for transplantation trials in animal models.

In order to harvest stem/progenitor cells from the SVZ areas according to the buoyancy, a medium is needed for density gradient centrifugation. Several media were introduced and among them, colloid silica treated with adsorbed polymers was found to have the required isoosmotic, pH-neutral and high density solutions[4,15,17,18,22,23].

In humans and mice, several specific protein markers for neural stem cell were introduced. Using these specific markers, one can separate and confirm the stem cell through the flow cytometric analysis. Unfortunately, there is no such specific marker in rats. The cell characteristics of the harvested cells from adult rats were evaluated through the immunostaining with several specific markers[10,25,28].

Previous reports concerned with specific cell markers showed that, nestin is a progenitor/neural stem cell marker, GFAP is an astroglia and primitive cell marker, O4 and Gal-C is a oligodendroglia marker and β-tubulin III is a matured neuronal marker. Although the cultured cells are not purified neural stem cells it can conclude through the immunostaining, that a large part of these cultured cells are progenitor/stem cells[9,16].

bFGF-2 has long been known for its pleiotropic effects on neural progenitors, and many groups have noted that bFGF-2 is a necessary nitrogen for maintaining the proliferative multipotent precursors in vitro. However, little is known regarding the potential of bFGF-2 to alter the lineage potential of a cell[3]. A developmental change in the bFGF-2 concentration might play a role in regulating the fate of neural stem cells. bFGF-2 expression in vivo is up-regulated concurrently with the switch of stem cells from a neuron-only program to one that also generates glia[17]. The in vitro, treatment of embryonic day 10 (E10) cortical progenitors with low levels of bFGF-2 (0.1ng/ml) retains cells in a neuron-only program. The treatment of these same cells with higher bFGF-2 concentration (10ng/ml) stimulates stem cell proliferation (i.e. the average clone size increase) and encourages the progeny to generate glia in addition to neurons. Although the authors argue that FGF-2acts to activate a gliogenic program, the observation that FGF-2 stimulates the production of multiple lineages in a population ordinarily limited to generating one cell type might actually be very similar to our findings. The obvious difference is that the main differentiation program of "adult"precursors are to generate glia and not neurons. If stem cells are involved in the adult gliogenic processes, perhaps both adult and embryonic precursors that are "normalized" to a multi-lineage program by high concentrations of FGF-2 might activate both neuronal and glial programs in the responding cells[17,24].

These results demonstrate that progenitor cells isolated from the adult rat SVZ and in vitro cultured with 20ng/ml of bFGF-2, retain the potential for survival and terminal differentiation into mature glia and neurons. In addition, the in vivo fate after the transplantation, these cells is clearly influenced by exogenous factors.

These cultured cells can survive and differentiate after the transplantation. A previous report showed that, various types of stem cells transplanted into an animal model survived and underwent differentiation, and migration[21]. Although these results are rather limited, this study shows that the transplanted cells survived and differentiated into neurons[1,20].

Conclusion

From this study, neural stem/progenitor cells can be harvested from an adult brain using a high-speed centrifugation method, and bFGF-2 can differentiate the cells into neurons

and glia. These cells can survive and differentiate in the new environment after transplantation.

However, a large scale study will be needed to confirm this.

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