Survival and Differentiation of Neuroepithelial Stem Cells following Transplantation into the Lateral Ventricle of Rats

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Abstract

Neuroepithelial stem cells (NEPs) demonstrate a high potential for self-renewal and differentiation during embryonic development. To explore the survival and differentiation of NEPs *in vivo*, we isolated NEPs from green fluorescence protein (GFP) transgenic embryos and transplanted into the lateral ventricle of rats. *In vitro* culture, NEPs proliferated into neurospheres and differentiated into both neurons and glia. When transplanted into the lateral ventricle of rats, these GFP positive NEPs (GFP+ NEPs) survived and attached to the wall of ventricle. Moreover, grafted cells differentiated into neuron-specific enolase (NSE) positive neurons and glial fibrillary acidic protein (GFAP) positive astrocytes and migrated into the host brain. Thus, our results indicate that NEPs can survive and differentiate into neurons and astrocytes in the lateral ventricle following transplantation.

Key Words: embryonic, neuroepithelial cells, lateral ventricle, transplantation

Introduction

Neural stem cells are multipotent cell lines with the potential of differentiating into neurons and glia, which holds great promise for cell therapy (6). Many experiments have focused on the signaling molecules controlling the differentiation of neural stem cells, and some of the studies have focused on exploring the feasibility in clinical application (12). Neuroepithelial stem cells (NEPs) which are produced earlier in development demonstrate an ability to differentiate into hundreds of different neuronal subtypes and many different glia (1). Our previous studies, and those of other investigators, showed that NEPs demonstrated high potential differentiation *in vitro* culture (15, 17).

NEPs are potential donor cells for medical application due to its ability of vigorous neuronal differentiation, not only for neurodegenerative disease described in our previous studies (13, 14), but also for many other neurological injuries. Recent studies reported that the lateral ventricle was the best site of transplantation of neural stem cells into the brain for treating hypoxic-ishemic damage (16). The grafted cells in the lateral ventricle grew better than those in the caudate parenchyma (7). It is likely that the lateral ventricle provide instructive environmental cues that

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allow donor cells to survive (18). We are wandering whether NEPs can survive and differentiate into neurons in the lateral ventricle and can be used as a potential strategy for the treatment of neurological disorders. To date, there have been very few studies on such a phenomenon. Here, we transplanted green fluorescent protein positive neuroepithelial stem cells (GFP^+ NEPs) taken from GFP transgenic embryos into the lateral vernticle of rats. Immunohistochemical staining was employed following transplantation at various survival times.

Materials and Methods

Animals

Experiments were carried out with Wistar rats (250-300 g) obtained from Shandong University. All procedures were approved by the Institutional Animal Care Committee, and were in accordance with the China Animals (Scientific) Procedures.

Cell Culture

GFP^+ NEPs were kindly provided by Dr. WS Poon (The Chinese University of Hong Kong, Hong Kong, PRC). GFP^+ NEPs were harvested from enhanced GFP transgenic mice (green mice), in which the expression of GFP is under the control of a chicken-actin promoter. A method was used as previously described (13). Briefly, NEPs were isolated from the neural tubes of GFP^+ transgenic embryos (embryonic day 11, 11th day of gestation), then suspended in N2 medium, a defined DMEM/F12-based medium (Life Technologies) supplemented with B27 (Gibco, Calsbad, CA, USA). Subsequently, NEPs were plated into uncoated six-well plates at a density of 3×10^4 cells/ml and passaged by mechanical dissociation every 7-10 days. The cells used for transplantation were collected from neurospheres of 5-8 passages and resuspended in serum-free culture medium at a final concentration of 2×10^7 cells/ml. For the differentiation assay, dissociated NEPs from spheres were plated onto poly-L-lysine coated coverslips and cultured for additional 10 days in DMEM/F12 containing 10% fetal bovine serum (FBS). For nestin staining, neurospheres were cultured for 2h. Before transplantation, trypan blue was added to a sample of cell suspension and viewed in a hemocytometer to assess cell viability and to determine cell counts. Cell suspensions of >90% viability was used for transplantation.

Transplantation of NEPs

Fifteen rats were randomly divided into three groups (n = 5) and were anesthetized with pentobarbital (50 mg/kg, i.p.). Five microliters of the suspension containing 1×10^5 cells was injected into the left lateral ventricle at the following coordinates (in mm, with reference to bregma and dura): anterior (A): -1. 0, lateral (L): 1.4, ventral (V): 3.8. The injection rate was 1 μl/min, and the cannula remained in place for an additional 5 min before retraction. Five rats from each group were sacrificed at 14, 21 and 28 days after transplantation, respectively.

Stainings for Cryosections and Cultured Cells

The rats were anesthetized with pentobarbital and perfused with 4% paraformaldehyde (PFA). Brains were cryoprotected in graded sucrose solutions and frozen. Sections were cut at 40 μm on a freezing microtome for different stainings. Both cultured cells and spheres were fixed in 4% PFA for 10 min before staining.

To evaluate the survival, distribution of grafted NEPs, 4',6-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, MO, USA) staining was performed. Briefly, sections were washed in phosphate buffer (PBS) for 10 min, and stained in a DAPI solution (1 μg/ml) for 5 min at room temperature. Subsequently, the sections were washed again in PBS, and then mounted with mounting medium for fluorescence (Vector Laboratories, Burlingame, CA, USA).

To identify the differentiation of cultured NEPs *in vitro* and transplanted NEPs *in vivo*, immunohistochemical staining was performed. Briefly, fixed cryosections and cultures were blocked in PBS containing 0.3% Triton X-100 and 10% donkey serum for 30 min at room temperature, followed by an overnight incubation in the same buffer at 4°C with one of the following antibodies: rabbit anti-neuron-specific enolase (NSE), rabbit anti-glial fibrillary acidic protein (GFAP), rabbit anti-galactocerebroside (GalC) (all diluted at 1:200, Sigma), mouse anti-Nestin (diluted at 1:500, Developmental Studies Hybridoma Bank, Iowa City, IA). After three washes with PBS, sections and cultures were incubated with secondary antibodies conjugated to Cy3 (Jackson ImmunoResearch, West Grove, PA, USA) diluted at 1:1000. Fluorescent reactivity was visualized by fluorescence microscopy (Zeiss Axiovert, Jena, Germany). Furthermore, the immuno-reactivity was also detected by incubations with biotinylated secondary antibodies and avidin-biotin complex (vectastain ABC kit, Vector Lab, Burlinhame, CA, USA), then visualized with diaminobenzidine (Sigma). Selected sections with which primary antibodies were omitted while secondary antibodies were used as controls to verify the specificity of staining patterns.

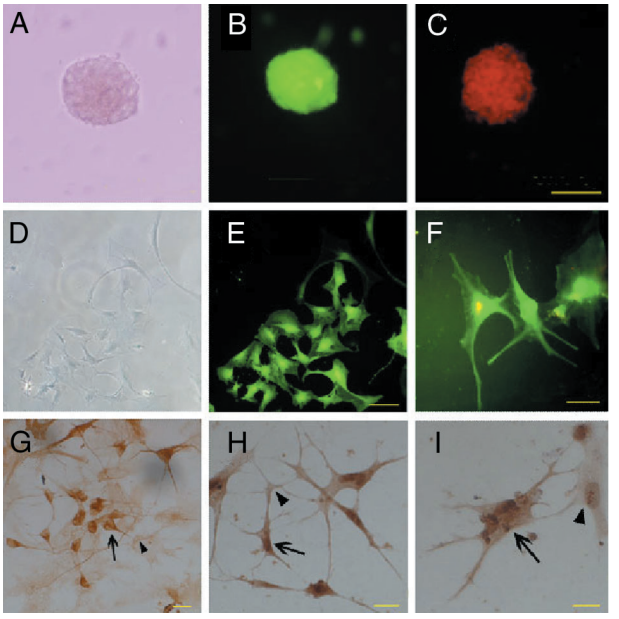

Fig. 1. GFP$^+$ NEPs in culture. NEPs formed spheres at 7 days in culture (A). The same image of A visualized under fluorescence microscope (B). Fluorescence immunostaining with antibody to nestin (C). At day 3 *in vitro*, cultured NEPs were visualized under microscope (D, E). Typical neuron-like cells were presented at day 10 in culture (F). At 10 days in culture, NEPs differentiated into NSE$^+$ cells (G, arrows), GFAP$^+$ cells (H, arrows) and GalC$^+$ cells (I, arrows), arrowheads denoted negative cells in G, H and I. Scale Bars: A-C, 100 μm; D-F, 50 μm; G-I, 20 μm.

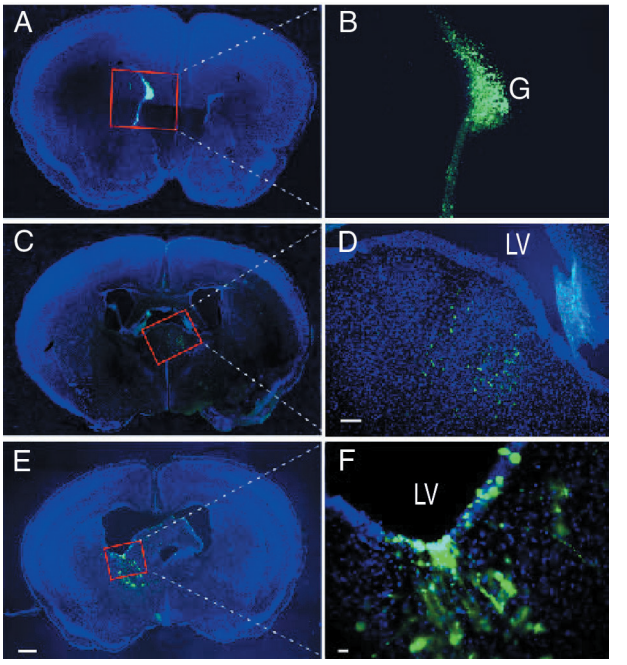

Fig. 2. Transplantation of GFP$^+$ NEPs into lateral ventricle. B, D and F were the high magnification of A, C and E, respectively. NEPs were injected into the lateral ventricle (A). GFP$^+$ cells in graft region were visualized under fluorescence microscope (B). Grafted cells attached the wall of lateral ventricle (E) and migrated into the host brain at 21d (C, D) and 28d (E, F) after transplantation. LV = lateral ventricle, G = graft. Scale Bars: A, C, E, 500 μm; B, D, 100 μm; F, 20 μm.

Results

In Vitro Characterization of NEPs

GFP$^+$ NEPs were cultured in DMEM/F12-based serum-free media. At day 7, abundant neurospheres were evident in culture and could be detected directly under the fluorescence microscope (Fig. 1, A and B). Lendahl *et al*. reported that nestin could distinguish the stem cells from the differentiated cells in the neural tube (4). We used the antibody to nestin as a marker to label neural stem cells. Undifferentiated spheres were all immuno-positive for nestin (Fig. 1C). When cultured in DMEM/F12 medium containing 10% FCS, NEPs cells exhibited green fluorescence under the microscope (Fig. 1, D and E). Typical neuron-like cells extended long neurites in culture (Fig. 1F). At 10 days *in vitro* culture, immunohitochemical staining showed typical NSE positive neurons (NSE$^+$), GFAP positive astrocytes (GFAP$^+$) and GalC positive oligodendrocytes (GalC$^+$) (Fig. 1, G-I).

Histological Staining Detects the Survival of Grafted NEPs

NEPs were stereotaxically injected into the lateral ventricle, grafted cells attached to the ventricular wall and survived well (Fig. 2, A and B). Of fifteen rats that received grafted NEPs, all of them had evidence of surviving cells. They formed a localized, dense cell grafted region. The clumps of donor cells were also observed in the ventricles. Under the fluorescence microscope, GFP$^+$ cells were clearly visualized. After attaching to the ventricle wall, transplanted cells migrated from the grafted region into the host brain following 21d and 28d transplantation (Fig. 2, C-F).

Immunohistochemical Staining Detects the Differentiation of Grafted NEPs

Histological immunostaining was employed to further confirm the differentiation of grafted cells. No NSE$^+$ cells could be detected at 2 weeks after transplantation. At 21d after transplantation, NSE$^+$ cells were detected in the lateral ventricle. More NSE$^+$ cells could be observed at 4 weeks post transplantation (Fig. 3A). Cells with short multipolar processes were evident (Fig. 3B). Similarly, using a specific antibody against

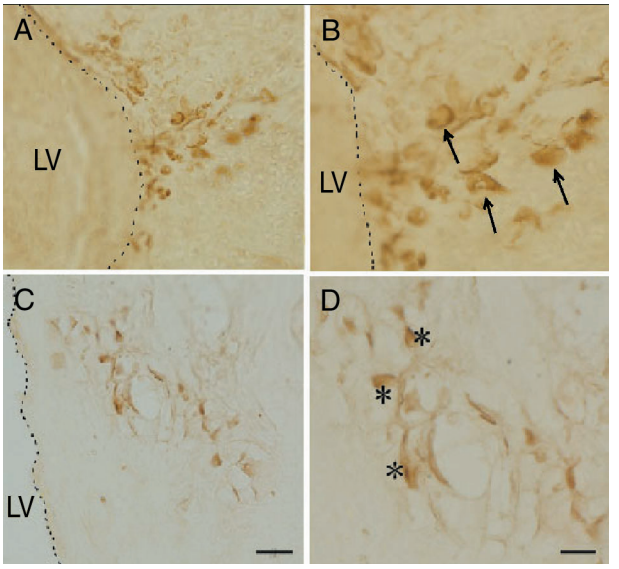

Fig. 3. Immunostaining of grafted NEPs with NSE and GFAP. B and D were the high magnification of A and C, respectively. NSE positive cells (B, arrows) and GFAP positive cells (D, asterisks) were detected at 28d after transplantation. LV = lateral ventricle, Scale Bars: A, C, 50 μm; B, D, 25 μm.

GFAP, we also detected a number of astrocytes in the lateral ventricle at 3 and 4 weeks after transplantation. $GFAP^+$ cells were observed with a short process in the grafted region (Fig. 3C), and different shapes of cells (unipolar, bipolar, and multipolar) were also readily identified in the grafted region (Fig. 3D). Moreover, we examined the distribution of NSE^+ and $GFAP^+$ in the host brain at 21d and 28d after transplantation. Many NSE^+ and $GFAP^+$ cells were found < 0.5 mm from the surface of lateral ventricle. Few NSE^+ cells could be found in the region of 0.5-1.0 mm except for a few $GFAP^+$ cells (Table. 1).

Table 1. Distribution of NSE and GFAP positive cells at 21d and 28d following transplantation into lateral ventricle*

	< 0.5 mm		0.5-1.0 mm	
	21d	28d	21d	28d
NSE^+ cells	++	+++	0	0
$GFAP^+$ cells	+++	++++	+	+

* NSE and GFAP positive cells were counted microscopically in randomly selected areas (at high magnification × 400) in immunostaining sections. The distance was measured from the edge of the ventricular surface. The data were based on observations from three representative animals in each group. 0, cells not found; +, 1-5 cells; ++, 6-10 cells; +++, 11-20 cells; ++++, > 20 cells.

Discussion

In this study, we have evaluated the survival and differentiation of NEPs following transplantation into the lateral ventricle of rats. The results showed that grafted NEPs survived and differentiated into both neurons and astrocytes in the host brain. A similar neuronal differentiation of NEPs was reported after transplantation into hippocampus (3). It was documented that lateral ventricle is a good site for transplantation (16). Probably, the nutritious factors in the cerebrospinal fluid make the lateral ventricle an implantation site favorable for NEP survival. Previous studies reported that both grafted fetal substantia nigra and cells from adrenal medulla survived well in the lateral ventricle (10, 11). Our studies also suggested that NEPs from neural tube could survive and differentiate into neurons in the lateral ventricle.

The current experiment also demonstrated that grafted cells could migrate into the host brain. Most differentiated NSE^+ and $GFAP^+$ cells were found < 0.5 mm from the surface of lateral ventricle. McMahon and McDermott (5) reported that transplanted cells in younger animals could migrate a greater distance from the ventricular surface than did those in older age. It is likely that various host microenvironments potentiate the selective migration of donor cells. Further studies are necessary to determine the relationship between microenvironment and donor cells survival and differentiation.

Recently, Kanunobu *et al.* (9) described the characters of NEPs in detail. They found that the expression of nestin in NEPs was downreglulated rapidly during neuronal differentiation. And, the expression pattern of nestin overlapped with the immunoreactivity of Musashi1, an RNA-binding protein that is highly enriched in CNS precursor cells (2). Moreover, nestin positive NEPs also expressed Ki67, which is expressed specifically in proliferating cells. These results implied that NEPs were neural stem cells. In our present study, NEPs from neural tube also demonstrated multipotency and had the capacity to self-renew. To easily distinguish donor-derived cells from the endogenous host neurons after transplantation, GFP^+ NEPs isolated from the enhanced green fluorescent protein transgenic mice were employed in this experiment. The advantage of using GFP as a reporter is that we could observe GFP fluorescence in live and intact cells (8).

In conclusion, our study demonstrates that NEPs derived from the neural tubes survived and differentiated into neurons and astrocytes. Also, grafted cells could migrate into the host brain. This finding suggests that NEPs could be used as a source for treating neurological diseases following transplantation into the lateral ventricle.

Acknowledgments

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