Regionspecific survival and differentiation of mouse embryonic stem cell-derived implants in the adult rat brain

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Abstract: Totipotent and regionally non-specified embryonic stem (ES) cells provide a powerful tool to understand mechanisms controlling stem cell differentiation in different regions of the adult brain. As the development capacity of ES cells in the adult brain is still largely unknown, we grafted small amounts of mouse ES (mES) cells into adult rat brains to explore the survival and differentiation of implanted mES cells in different rat brain regions. We transplanted the green fluorescent protein (GFP)-positive mES cells into the hippocampus, septal area, cortex and caudate nucleus in rat brains. Then the rats were sacrificed 5, 14 and 28 d later. Of all the brain regions, the survival rate of the transplanted cells and their progeny were the highest in the hippocampus and the lowest in the septal area \((P<0.01)\). The grafted ES cells could differentiate into nestin-positive neural stem cells. Nestin-positive/GFP-positive cells were observed in all brain regions with the highest frequency of nestin-positive cells in the hippocampus and the lowest in the medial septal area \((P<0.01)\). mES cells differentiated into end cells such as neurons and glial cells in all transplantation sites in recipient brains. In the hippocampus, the ES cells differentiated into neurons in large amounts. These results demonstrate that only some brain regions permit survival of mES cells and their progeny, and form instructive environments for neuronal differentiation of mES cells. Thus, because of regionspecific presence of microenvironmental cues and their environmental fields, the characteristics of the recipient tissue were considerably important in formulating cell replacement strategies for neural disorders.

Key words: survival; differentiation; embryonic stem cell; cell transplantation; rat
There is evidence from both animal models and clinical investigations showing that fetal dopamine (DA) neurons can produce symptomatic relief in some younger patients with Parkinson’s disease (PD)\[1\]. Cell transplantation is proved to be feasible to treat the central nervous system (CNS) diseases. The ability of the transplanted neural stem cells (NSCs) to differentiate into neurons or glia varies greatly among distinct regions of the CNS. NSCs differentiate principally into glia when transplanted into the cerebellum, striatum and spinal cord\[2-5\]. Hippocampus-derived progenitors differentiate into olfactory neurons when grafted in the rostral migratory stream\[6\]. The phenotypic fate of transplanted neuronal precursor cells is affected by the selective hippocampal lesions, which suggests the presence of some endogenous neurons and the partially conserved cytoarchitectural organization are essential for neuronal precursor cells to differentiate into region-specific neuronal cell types\[7\]. In vitro studies using astroglia-conditioned medium and astroglia/NSC co-culture suggest the importance of the local microenvironment for survival and differentiation of stem cells in the brain\[8-10\]. Embryonic stem (ES) cells are multipotent progenitors with unlimited developmental potential. Totipotent mouse ES (mES) cells can be maintained in an undifferentiated, proliferative state in the presence of leukemia inhibitory factor (LIF). When LIF is removed, these mES cells differentiate into a variety of cell types in vitro, spontaneously or via inductive agents\[11-15\]. When transplanted into the CNS, the ES cell-derived embryoid bodies and ES cell-derived neural cells differentiate into neuronal lineage cells\[16,17\]. Totipotent and regionally non-specified ES cells provide a powerful tool to study stem cell differentiation in different regions of the adult brain. It remains yet to be determined whether growth of ES cells in distinct regions of the adult brain follows a uniform pattern, or whether some areas are superior in promoting development of ES cells. In this study, we have determined the influence of the recipient brain regions on the commitment of undifferentiated ES cells to a neural fate, and the survival and differentiation of transplanted ES cells.

1 MATERIALS AND METHODS

1.1 mES cell culture and green fluorescent protein (GFP) expression

mES cells of the D3 cell line (obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Science), stably transfected with the pCX-EGFP expression vector by Lipofectamine 2000 (Gibco, Invitrogen Corporation, USA) were cultivated on mitotically inactivated SNL fibroblasts (permanent line of irradiated mouse fibroblast cells, obtained from Institute of Genetics and Developmental Biology, Chinese Academy of Science) in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Invitrogen Corporation, USA) containing 15% fetal calf serum (FCS) (Hyclone, USA), non-essential amino acids (Hyclone, stock solution 1:100, USA), penicillin-streptomycin (Hyclone, stock solution 1:100, USA), 2-mercaptoethanol (Sigma, 100 µmol/L, USA) and L-glutamine (Hyclone, 2 mmol/L, USA), at 37 °C in a humidified atmosphere of 5% CO₂. The cells were subcultured every 2 d. Selection was performed using G418 (Gibco, Invitrogen Corporation, USA) to identify clones which had incorporated the transfected genes.

1.2 Preparation of single cell suspension for implants

GFP-mES cells were cultured on gelatin-coated dishes for two passages in the presence of mouse recombinant LIF (1 000 U/mL, PeproTech, UK) prior to transplantation in order to separate mES cells from co-cultured mouse fibroblasts. ES cells were collected, washed in phosphate-buffered saline (PBS) and allowed to settle by gravity. ES cells were then centrifuged at 1 000 r/min, resuspended in DMEM and allowed to stand for 5 min to make cell clumps settle. The remaining single cell suspension was diluted to a concentration of either 4×10⁴ or 4×10⁵ cells/10 µL. Approximately 85%-90% of all transplanted cells were mES cells as determined, and their undifferentiated state was verified by staining of mES colonies for alkaline phosphatase (AKP) and mouse anti-stage-specific embryonic antigen 1 (SSEA-1, 1:10, Chemicon, USA).

1.3 Transplantation, immunosuppression and post-transplantation monitoring

Adult female Sprague-Dawley rats (180-220 g) were used in transplantation experiments. To reduce immune reaction of the host brain and subsequent graft rejection, all animals received immunosuppression by subcutaneous injection of cyclosporin A (Novartis, 10 mg/kg, Switzerland) diluted in physiological saline each day throughout the survival period. Animals were anaesthetized with 10% chloral hydrate (3.5 mL/100 g) and the head position was secured in a stereotaxic frame equipped with a rat adaptor. Single cell suspension of mES cells was infused in the medial septum (AP +0.9 mm; L +0.5 mm; DV +6.5 mm), cortex (AP -2.0 mm; L -2.0 mm; DV +1.5 mm), hippocampus (AP -3.2 mm; L +3.0 mm; DV +3.0 mm), or caudate...
nucleus (AP -0.2 mm; L +3.0 mm; DV +5.8 mm), at a final volume of 10 µL. mES cells were grafted within 5 min with a 20 µL microsyringe that was left in place for 10 min post-infusion to allow mES cells to settle before needle removal. In 4×10^4 cells/10 µL group we grafted cells merely in the hippocampus or caudate nucleus to minimize the number of animals, and in 4×10^4 cells/10 µL group we grafted cells in all four locations. The control group was transplanted with PBS. After transplantation, the rats were returned to their home cages and allowed to recover until regaining consciousness and proper movement control. Body weight of the rats showed transient reduction within 48 h post-operation followed by a gradual increase.

1.4 Histological procedures
Five, 14 and 28 d after transplantation, animals were terminally anesthetized with 10% chloral hydrate (3.5 mL/100 g) then perfused intracardially with 100 mL of 0.9% saline and followed by 400 mL of paraformaldehyde (4% in PBS). The brains were removed and postfixed for 8 h in the same 4% paraformaldehyde solution. Following postfixation, the brains were equilibrated in sucrose for 48 h at 4 °C (30% in PBS). Series of 10-μm thick serial coronal sections were cut on a cryostat microtome. The routine method was used for Nissl’s staining. We ensured random systematic sampling of each graft.

1.5 Immunohistochemistry and cell counting
Sections were rinsed in PBS, preincubated in 4% normal goat serum for 30 min, and incubated overnight at room temperature with mouse anti-glia fibrillary acidic protein (GFAP, BD, 1:50), mouse anti-neuron-specific nuclear protein (NeuN, 1:100, Chemicon), mouse anti-nestin (Chemicon, 1:50), and rabbit anti-proliferating cell nuclear antigen (PCNA, Proteintech, 1:300), diluted in PBS with 2% normal goat serum and 0.1% Triton X-100. After rinse in PBS, sections were incubated in fluorescence-labeled secondary antibodies Cy3 (Proteintech, 1:100) or RPE (Serotec, 1:50; only for SSEA-1) in PBS with 2% normal goat serum and 0.1% Triton X-100 for 30 min at room temperature. Fluorescence staining was evaluated by using an Olympus fluorescence microscope. Omission of primary antibodies in the control experiments resulted in the lack of any cellular labeling. Sections used for cell counting were labeled with 10 μg/mL (in PBS) bisbenzimide (Hoechst 33342, Sigma). Hoechst 33342 was performed on sections over 15 min, and followed by rinsing in PBS. Nuclei were stained with Hoechst 33342. The presence of a Hoechst-labeled nucleus in a GFP-expressing cell enabled us to distinguish between adjacent donor cells and to obtain the total number of donor cells found in each section. The number of cells expressing a particular cell marker was counted and compared to the total number of donor cells found in each section. Counting of GFP-positive neurons were performed on every sixth section and measured on fourteen randomly. The ratio of nestin-positive cells was measured on ten randomly.

1.6 Statistical analysis
Statistical analysis was performed by using Statistical Product and Service Solutions (SPSS) 12.0 software.

2 RESULTS

2.1 Establishment and characterization of GFP-positive mES cells
Mouse D3 ES cells constitutively expressing GFP were established by Lipofectamine 2000 with an expression construct and subsequent selection of G418 resistant clones (Fig.1). Individual undifferentiated, AKP-positive clones (Fig.2) were allowed to differentiate and to form embryoid bodies (Fig.3). Neurospheres and neural progenitors were generated from embryoid bodies and placed in differentiation medium where they gave rise to all major types of neural progeny including differentiation to cells with an apparent neuronal morphology. A SSEA-1- and AKP-positive mES clone which retained GFP expression in neurons and GFAP-positive astroglial cells after in vitro differentiation was selected. We then performed transplantation of mES cells into the medial septum, caudate nucleus, cortex and hippocampus of adult female rats. The cells were stained for AKP and SSEA-1 to confirm the transplantation of undifferentiated mES cells (Fig.2). mES cell grafts in all brain regions led to the development of cells with neuron and glia-like morphologies, as detected by fluorescence detection of GFP.

2.2 Survival and growth of transplanted mES cells
Transplanted mES cells were detected in the rat brains that were harvested 5, 14 and 28 d post-transplantation. No GFP-positive cells were observed in any PBS-injected rat brains. All ES cell grafts exhibited a slightly higher cell density than the surrounding host brain tissue, as indicated by Nissl’s staining (Fig.4). The GFP expressing mES cells of the D3 cell line also retained GFP expression after being grafted into the adult rat brain (Fig.5). Most survival cells remained to localize near the injection track and some migrated. In 4×10^4 cells/10 µL transplantation group the mES cells could be detected in all rat brains. Graft sizes and overall histological appearance varied greatly among
animals. The diameter of the largest graft was approximately 3 mm. The graft exhibited complex tissue types with significant fractions of graft volume that were negative for neuronal markers. The graft also contained regions which were stained positive for PCNA. Large parts of the grafts were necrotic, reflecting a mismatch between cell proliferation and angiogenesis. In $4 \times 10^4$ cells/10 $\mu$L transplantation group, surviving transplants were found in most rats. Graft sizes and overall histological appearance were similar. The graft diameters were shorter than 1 mm. The PCNA-positive regions were only present in the grafts 5 d post-transplantation. Survival cells distributed near the injection location and migrated along. Necrotic zones were seldom found in the grafts. The number of survival cells in the septal area was much lower than that in any other graft sites 28 d post-transplantation ($P<0.01$). Though some cells in the hippocampus and cortex appeared atrophic, total survival cell number in hippocampus was higher than that in other sites ($P<0.01$). The graft cells here were agglomerate. In the caudate nucleus the transplanted cells scattered and the migration length was long. The cells survived well (Fig.6). In the caudate nucleus and cortex the
living cell number was similar ($P=0.443$).

2.3 Differentiation of mES cells to neural progenies varies among different brain regions

To explore the potential of different brain regions to provide permissive environments for mES cells to generate neural progenitors, we investigated whether mES cells ($4 \times 10^4$ cells/10 $\mu$L, 2 weeks after graft) can give rise to nestin-positive neural progenitors. Five days post-transplantation, nestin-positive cells were observed in all involved brain regions, but most of them were host-derived responsive astrocytes (GFP-negative and GFAP-positive), especially in the septal area. Fourteen and 28 d post-transplantation, nestin-positive/GFP-positive cells were observed in all brain regions, with the highest number of nestin-positive cells in the hippocampus ($P<0.01$) and the lowest number in the medial septum ($P<0.01$) (Fig. 5A, Table 1). These data suggest that the ratio of the transplanted mES cells differentiated to neural progenitors varies greatly among different brain regions (Table 1).

2.4 ES cell-derived neural progenies differentiate into mature neurons

While transplanted GFP-positive cells were immunonegative for any of the mature neuronal markers 5 d post-transplantation, the mES cell-derived NeuN-positive neurons (Fig. 5B) and GFAP-positive gliacytes (Fig. 5C) were identified 14 d and 28 d post-transplantation. The number of mES cell-derived NeuN-positive neurons was much higher in the hippocampus than that in other graft locations. Many GFP-positive cells in the medial septum appeared atrophic. Only a few GFP-positive cells could be detected in the rat brains 28 d post-transplantation, and the ES cell-derived NeuN-positive neurons were fewer. The number of gliacytes was high and most of them were GFP-negative. In the caudate nucleus, the cells dispersed, and the peripheral cells in the grafts shrank. The mES cell-derived neurons were divided by gliacytes. In the cortex, the ES cell-derived cells migrated along the callosum and most of them were neurons. The number of living cells was highest in the hippocampus among all the transplantation sites, and there were differentiated neurons around molecular layer of the dentate gyrus with few gliacytes mixed.

3 DISCUSSION

3.1 Survival and growth of mES cells in rat brains

The significant species difference between the host animal and donor cells may influence graft survival. Previous studies have shown that implantation of mouse embryonic neural tissue into the brain of guinea pigs resulted in no surviving grafts, in contrast to poorly surviving grafts observed when implanted into rats\textsuperscript{18}. In this same study, transplantation of rat embryonic neural tissue resulted in good survival in both implanted rat and guinea pig brains. This study serves to highlight the importance of species differences in animal models. Here we grafted undifferentiated mES cells into adult rat brain regions and the cells survived. The survival partially reflected the low immunogenicity of the mES cells. The cell number may also influence graft survival as described previously. It was because of immune reactions that high dose cell transplantation led to poor survivorship. The graft cells grew well in the low
dose group, and there were some PCNA-positive cells among them, indicating these cells had reproductive activity. The survival cell number in the septal area was obviously reduced 28 d post-transplantation, though the number in the neurogenesis area, e.g. hippocampus was still high. In the caudate nucleus and cortex the living cell number was similar (P=0.443). Most of the mES cells differentiated into neural progenitors and neurons 28 d post-transplantation. The cell survival differences among four transplanted locations could attribute to the distinct local environment in the various brain regions supporting neural progenitors and neurons differentiated from mES cells. Thus, the neurogenesis areas contain cues that either instruct neuronal differentiation and/or promote their survival. The septal area, which is transfixed by multiple nerve fibers, probably contains fewer cues. For this reason the cells transplanted here didn’t grow well.

3.2 Differentiation of mES cells to neural progenies varies among different brain regions

Initial state, totipotency, and normal developmental and functional capacities are all known for the ES cells used in the present study. The ES cells are capable of normally differentiating into a variety of cell types in vitro, including neural stem cells and neural progenitor cells[10]. These cells can further differentiate into various mature neural cell types, such as neuroglia cells and neurons[12-14], including employable and conclusive dopaminergic neurons[19,20]. Transplantation of cells firmly committed to a neuronal lineage and expressing immature or mature neuronal markers have a great propensity for neuronal differentiation in non-neurogenic brain regions[21]. Similar results have been obtained by transplanting the ES cell-derived embryoid bodies and ES cell-derived in vitro-differentiated neural cells[15-17,22,23]. Our study suggests that mES cells can give rise to neural progenitors in several regions of the adult rat brain.

We grafted undifferentiated mES cells into various adult mouse brain regions. Fourteen days post-graft, the number of ES cell-derived nestin-positive cells was the highest in the hippocampus and the lowest in the septal nucleus. The medial septum, a non-neurogenic area in the brain, exhibited a very poor ability to direct ES cells to differentiate into nestin-positive neural progenitors, and those that appeared atrophic. These data were not enough to demonstrate the neurogenic areas, such as the hippocampus, offered agents to promote ES cells to differentiate into neurons. However we could be certain that the neurogenic areas provide profitable environments for neural progenitor and neuron growth and development. We believe that the investigation and control of the local microenvironment were very important in transplantation treatments.

3.3 mES cells differentiate into end cells in the recipient brains

It is critical to find an efficient transplantation method in cell transplantation investigation. Lundberg et al. observed that when transplanted into adult brains, the plasticity of neural stem cells (generated from the embryonic striatum or hippocampus) was more restricted: a majority of these cells differentiated into glia[24]. Using expanded fetal ventral mesencephalic (VM) precursors in vivo resulted in low survival rate of 3%-5% of the grafted DA neurons, which eliminated the actual gain by in vitro cell number expansion compared with fresh (unexpanded) fetal day-12 VM[25]. After epidermal growth factor (EGF)-responsive progenitors isolated from the embryonic mesencephalon or striatum were transplanted into the adult striatum, Svendsen et al. observed low cell survival within small grafts, with few differentiated cells expressing neuronal markers[26]. In our present study the ES cell-derived cells were detected in each graft location. Some clumps of ES cell-derived neurons were detected in the hippocampus with few gliocytes mixed, indicating that the ES cells could differentiate into neurons in large amounts in rat brain. We conclude that ES cells could survive well and differentiate into considerable neurons in some regions of the adult brain, i.e., the hippocampus. These results suggest that the hippocampus: (1) releases factors to stimulate the expansion of ES-derived neural progenitors and promote neuronal differentiation; (2) does not contain the factors inhibiting the neuron survival and differentiation that probably exist in other regions of the brain; (3) constructs niches that are good for neurons to live. It is important to make further studies to know the key regulatory factors so as to control them in transplantation therapies.

In conclusion, the cells in the proper stage of development, the optimal number of cells, the best mode of cell delivery and the means to suppress or prevent the immune rejection of transplant need to be emphasized in transplantation treatments. To determine key microenvironmental cues in the adult brain was an integral part of successful transplantation strategies. The intervention of these cues could raise the survival rate of grafts. An alteration of the local environment could also be expected under pathological conditions in the brain, when cell replacement is explored as a possible treatment strategy. The lack or imbalance of environmental factors might also be considered in forming neurodegenerative disorders by inhibiting adult neurogenesis in the affected brain areas. Regulations of these factors are promising to cure these diseases, and the...
ES cells undoubtedly provide a powerful tool.

REFERENCES


