Targeted migration and differentiation of engrafted neural precursor cells in amyloid β -treated hippocampus in rats

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Abstract: Objective To observe the migration and differentiation of the neural precursor cells (NPCs) that derived from murine embryonic stem cells (ESCs) when they were transplanted into amyloid β (A β)-treated rat hippocampus. **Methods** MESPU35, a murine ESC cell line that express the enhanced green fluorescent protein (EGFP), was induced differentiation into nestin-positive NPCs by modified serum-free methods. The A β plaques and the differentiation of the grafted cells were observed by immunofluorescent staining. **Results** Comparing 16 weeks with 4 weeks post-transplantation, the migration distance increased about 5 times; the rate of migratory NPCs differentiating into glial fibrillary acidic protein (GFAP)-positive cells kept rising from (30.41 $\pm$ 1.45)% to (49.25 $\pm$ 1.23)%, and the rate of NPCs differentiating into neurofilament 200 (NF200) positive cells increased from (16.68 $\pm$ 0.95)% to (27.94 $\pm$ 1.21)%. Meanwhile, the GFAP-positive cells targeting to the ipsilateral side of A β plaques increased from 60.2% to 81.3%, while the NF200-positive cells increased from 61.3% to 84.1%. The migration distance had significant positive linear correlations to the neuronal differentiation rate ($r = 0.991$) and to the astrocytic differentiation rate ($r = 0.953$). **Conclusion** Engrafted NPCs migrate targetedly to the A β injection site and differentiate into neurons and astrocytes.

Keywords: embryonic stem cells; amyloid β peptide; cell transplantation; differentiation; migration; rat

1 Introduction

During the past decade, stem cell research has brought hopes to several neurodegenerative disorders such as Parkinson disease and Alzheimer disease (AD)^[1-3]. Embryonic stem cells (ESCs), which are derived from the inner cell mass of the mammalian blastocyst, have the capability to differentiate into all three embryonic germ layers^[4]. These self-renewing and totipotent cells represent a potentially unlimited donor source for tissue transplantation^[5]. Prompted extensive researches have been done on the possibility of using ESCs for cell replacement in neurodegenerative diseases in central nervous system

(CNS)^[6].

Neural precursor cells (NPCs) are differentiated from ESCs and could be a suitable candidate for cell replacement, cause they have the properties of neural stem cells (NSCs) such as self-renewal capacity and differentiation into neurons and glial cells^[7]. Engrafted NSCs are capable of residing in specific areas of the CNS, such as the subventricular zone (SVZ)^[8] and the dentate gyrus of the hippocampus, the two neurogenic regions known in adulthood^[9]. However, neuronal differentiation rate of the transplanted NSCs are too low in the host CNS to accomplish the functional recovery^[10]. Moreover, neuronal replacement make effect only if the proliferating or the transplanted NSCs and their progeny can migrate to the exact regions where neuronal replacement is required^[11].

Migration of neurons in the CNS takes place both during development and in adult neurogenesis. In adult intact, neurogenesis occurs in the subgranular zone of the den-

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tate gyrus. New neurons are born in the underlying subgranular layer of the hippocampus and migrate into the granule cell layer to become mature granule neurons^[12,13]. NSCs in the CNS also migrate toward the lesion sites in response to injuries^[14,15]. Cultured NSCs also show an extraordinary migratory ability *in vitro* and *in vivo*. However, little is known about the migratory direction of ESC-derived NPCs after they are grafted into the amyloid β (A β)-damaged hippocampus, nor the relationship between the migration and the neural differentiation of grafted NSCs.

The murine ESC cell line MESPU35^[16] expresses enhanced green fluorescent protein (EGFP) and could be distinguished between transplant and host cells. We attempt to investigate the post-transplantation characteristics of the ESCs induced NPCs *in vivo* and to observe the behavior of NPCs in A β -treated hippocampus.

2 Materials and methods

2.1 Animal Adult male Wistar rats (200–220 g) used in this study were supplied by the Animal Breeding Center of Third Military Medical University, China. All rats were housed under a standard light-dark condition (12/12 h light/dark cycle) with free access to food and water. All experimental procedures were performed in accordance with approved principles of laboratory animal care.

2.2 Feeder cells preparation and differentiation of ESCs *in vitro* According to our modified serum-free culture method^[17,18], murine embryonic fibroblasts (MEFs) were isolated from 13.5-d mouse embryos and expanded in the Dulbecco's modified Eagle's medium (DMEM) with high glucose (Gibco), supplemented with 10% fetal calf serum (Hyclone). The MEFs from passage 2 to passage 5 were used as feeder cells. The ESC cell line MESPU35 was dissociated into a single-cell suspension and plated onto unattached 70-mm bacteriological dishes to form embryoid bodies (EBs). The 4-d EBs were plated onto the adhesive tissue culture surface, and treated with poly-L-ornithine (15 mg/mL), laminin (5 mg/mL) and fibronectin (10 mg/mL) for 24 h. The medium was then replaced by the serum-free defined culture medium for NPC selection. After 5–7 d of selection, the nestin-positive cells were expanded for 6 d.

2.3 Establishing an A β -induced hippocampus damage model of rat According to previous reports^[19,20], A β 1–40 peptide (Sigma) was dissolved in sterile distilled-water at a concentration of 2 μ g/ μ L and incubated at 37 °C for 7 d to obtain fibrillary aggregation. Rats were anesthetized and

fixed in a stereotaxic apparatus (Shanghai, China). An area of skin on the top of the skull was shaved and sterilized conventionally. One small hole was drilled in the parietal bone posterior to the bregma on the right side of the midline, with coordinates at –3.1 mm anteroposteriorly, $\pm$ 1.9 mm mediolaterally relative to the bregma, and –3.5 mm dorsoventrally below the dura^[21]. The A β 1–40 solution was injected into the small hole at the rate of 1 μ L/min over 5 min by a microinjector. Additional 15 min were allowed before retraction of the needle (1 mm/min). Saline injection was used as control. A β plaques in the hippocampus were identified by immunohistochemistry at 2 weeks post-injection.

2.4 Cell transplantation Four weeks after injection of A β 1–40 peptide, fluorescence activated cell sorter (FACS)-sorted NPCs (approximately 1×10^6 cells/ μ L in PBS medium) or vehicle medium PBS (sham surgery control, 1 μ L; $n = 6$) was transplanted to the A β lesion site of rat at the speed of 1 μ L/min via a 10- μ L Hamilton syringe. All NPCs recipients and control rats were given cyclosporine A daily (10 mg/kg p.o., Novartis Pharmaceuticals Co.).

2.5 Calculation of migration distance and differentiation rate After transplantation, the surviving cells within the migrating area were evaluated. A 1-in-5 series of coronal sections (20- μ m thick) in the range from 580 μ m rostral to 580 μ m caudal to the injection site were collected, for all EGFP-positive transplanted cells resided in this range^[22]. Dead cells and red blood cells, which also have autofluorescence reaction under a fluorescence microscope, were excluded according to the methods of Muraoka *et al*^[22]. Those green/red double positive cells were considered as dead cells or red blood cells; only the green positive while red negative cells were considered to be the surviving cells.

The rate of engrafted NPCs differentiating into astrocytes (or neurons) was obtained by comparing the number of glial fibrillary acidic protein (GFAP)/EGFP dual-positive cells (or NF200/EGFP dual-positive cells) with the surviving EGFP-staining cells in the grafted field (totally 5 sections for each rat). The distance of engrafted NPCs migrating from the core of needle tract to the migrating positions were measured using the SPOT Insight software (Diagnostic Instruments, Michigan, USA).

2.6 Immunofluorescent staining Rats were transcardially perfused and dehydrated. The coronal sections were cut at 20 μ m on a cryostat (Leica). Free floating sections were rinsed with 3% H₂O₂ for 30 min to block the endogenous peroxidase activity, and then washed with 0.01 mol/L PBS

for 10 min for three times. Being blocked with goat serum (from the ABC Kit) for 60 min, sections were incubated with rabbit polyclonal anti-GFAP antibody or mouse polyclonal anti-NF200 antibody (both 1:100 dilution, from Beijing Zhongshan Biotechnology Co. Ltd., China) at 4 °C overnight, then incubated with ABC-Cy3 (1:100, Wuhan Boster Co., China) for 2 h at room temperature. The deposition of A β injection were detected by incubation with anti-A β antibody (1:1 000, Sigma) and Cy5-conjugated goat anti-mouse IgG (1:500, Wuhan Boster Co., China). Negative controls included all immunohistochemical procedures only without adding primary antibodies. Fluorescent signals were observed under a fluorescence microscope.

2.7 Statistical analyses Data is expressed as mean $\pm$ SEM. Statistical analysis of the data was performed by using ANOVA followed by Student Newman-Keuls tests and correlation analysis. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Differentiation of murine ESCs into NPCs *in vitro*

ESCs were plated on the replication-arrested MEFs and proliferated as colonies. The EBs were attached and the proliferating cells were spread and expanded. During the first 72 h in N2 plus fibronectin medium, a large proportion of cells were detached from the plate and lysed. The remaining cells changed their morphology from tightly packed epithelial cells to small elongated cells. The cells still expressed EGFP and were nestin-positive (results shown in our previous report^[17]).

3.2 Migration of the engrafted NPCs in A β -treated hippocampus At 4 weeks post-transplantation, most of engrafted NPCs were aggregate along the injection needle tract, only a few EGFP-positive cells spread away from the injection tract with very short distance (Fig. 1A). The NPCs remained in a cluster and possessed a round-shaped morphology. At 16 weeks post-transplantation, the number of migratory cells increased significantly and the cells dispersed in various directions, some cells could be observed in the dentate gyrus. Most of the migratory cells were single dispersed while a few cells were assembling (Fig. 1B). The average distances of cells migrating from the

Fig. 1 Migration of the engrafted NPCs in the hippocampus damaged by A β . Photomicrographs of the distribution and the dispersed migration of the engrafted NPCs expressed EGFP apart from the needle tract at 4 weeks (A) and 16 weeks (B) post-transplantation; and the measurement of the distance by the software. White arrows show the NPCs injection needle tract. Scale bar, 100 μ m. C: Comparative analysis of migration distance in different stages post-transplantation. * $P < 0.05$.

injection tract were (23.46 ± 2.53) , (50.53 ± 1.86) , (93.03 ± 2.77) , and (119.27 ± 3.62) μm at 4, 8, 12, and 16 weeks post-transplantation, respectively. The NPC migration distance increased by almost 5 times at 16 weeks compared with that at 4 weeks post-transplantation (Fig. 1C). No EGFP-positive cells were observed in the sham surgery group.

3.3 Differentiation of the engrafted-NPCs *in vivo* At 4 weeks post-transplantation, the majority of grafted cells remained at the transplantation site, and neither demon-

strated morphologic changes nor expressed phenotype-specific markers. A few cells migrated away from the needle tract, and these NF200-positive cells lost their former round shape and had short processes (Fig. 2A-C). More migratory grafted cells expressed GFAP (Fig. 2D-F). At 16 weeks post-transplantation, the proportion of NF200-positive cells among the migratory cells increased significantly. They had an almost mature morphological feature with clear cell bodies, and long and thick processes. These new born

Fig. 2 At 4 weeks post-transplantation, some grafted cells (EGFP-expressed, showed as green cells) migrated from the needle tract and were NF200-positive (cy3-ABC, showed as red positive cells); short processes were also observed in these cells (A, B, C). Some migratory grafted cells were GFAP-positive (D, E, F). Most of differentiated cells were localized on the ipsilateral side to A β -deposition (Cy5-conjugated goat anti-mouse A β IgG, showed as blue plaques) at 4 weeks post-transplantation (C, F). At 16 weeks post-transplantation, the number of NF200-positive cells among the migratory grafted cells increased and they appeared almost mature, with clear cell bodies and long, thick processes (G, H, I). Most of the engrafted NPCs that migrated from the needle tract were GFAP-positive. These astrocyte-like cells inclined to stretch out evictions to overlap with other cells (J, K, L). Most of NF200 positive cells and GFAP-positive cells distributed on the same side of A β -deposition at 16 weeks post-transplantation (I, L). Scale bar, 50 μm .

NF200-positive cells also inclined to extend their axons to the target areas (Fig. 2G-I). Most of the engrafted NPCs that migrated away from the needle tract were GFAP-positive. They lost their round shape and were ramified with bigger cell bodies and longer evecton. These astrocyte-like cells inclined to stretch out evectons to overlap with other cells (Fig. 2J-L).

For the migratory cells outside the luster, the differentiation rates of GFAP-positive cells and NF200-positive cells were $30.41\% \pm 1.45\%$ and $16.68\% \pm 0.95\%$, respectively, at 4 weeks post-transplantation. Twelve weeks later (at 16 weeks), the rates increased to $49.25\% \pm 1.23\%$ and $27.94\% \pm 1.21\%$, respectively (Fig. 3). However, the phenotype-specific marker was not detected in most of the engrafted cells remaining within the transplantation site.

Fig. 3 Average rates of the migratory NPCs differentiating into the GFAP-positive cells and NF200-positive cells at 4, 8, 12, and 16 weeks post-transplantation. * $P < 0.01$, ** $P < 0.001$.

The GFAP-positive cells which were differentiated from grafted NPCs distributed asymmetrically: 60.2% of them were localized on the ipsilateral side to the A β deposition at 4 weeks post-transplantation (Fig. 2F), and this percentage significantly increased to 81.3% twelve weeks later ($P < 0.01$) (Fig. 2L). As to the distribution of neuronal differentiated cells, 61.3% of which were observed on the same side of A β deposition at 4 weeks post-transplantation (Fig. 2C), and the percentage markedly increased to 84.1% at 16 weeks post-transplantation (Fig. 2I) ($P < 0.01$).

3.4 Correlation analysis between the migration distance and the differentiation rate of the migrating engrafted-NPCs The correlation between the migration distance and the rate of the migratory cells differentiating into astrocytes or neurons was evaluated at 4, 8, 12 and 16 weeks post-transplantation. A significant positive linear correlation

existed between the migration distance and the differentiation rate of neuron-like cells ($r = 0.991$, $P = 0.009$), and between the migration distance and the differentiation rate of astrocytic-like cells ($r = 0.953$, $P = 0.047$) (Fig. 4).

Fig. 4 Correlation analysis between migration distance and differentiation rate of the migratory engrafted-NPCs at 4, 8, 12, and 16 weeks post-transplantation.

4 Discussion

The basic principle of cell therapy is to restore the lost brain functions due to damages or diseases by replacing the dead cells with healthy cells through transplantation^[2]. One possible mechanism by which the engrafted cells exert functions may be re-forming the lesion-damaged neuronal networks. It has recently been reported that neurons derived from NSCs are able to promote the re-formation of neuronal networks *in vitro* and to form the functional synapses that generate action potentials, supporting the idea that neurons derived from *ex vivo* cell sources may be able to integrate into neuronal circuitries^[23]. Neuronal replacement will be possible only if the grafted cells and their progeny can migrate within the CNS and reach the precise regions where neuronal replacement is required^[24]. In addition, NPC transplants can accelerate the proliferation and differentiation of the NSCs in the hippocampus, which also may produce a reproducible therapy.

We observed in the present study that the NPCs derived from murine ESCs migrated targetedly toward A β injection locations when they were grafted into the hippocampus of rats. The migration is very limited compared with former reports, in which the maximal migration distance was attained by some specified cells originated from the homotopically and homochronically transplanted solid pieces of adult SVZ of rats. These cells that migrated for several millimeters along the rostral migratory stream (RMS), were able to reach the olfactory bulb^[25,26]. However, others

found that those NSCs isolated from mouse SVZ fail to migrate and acquire their normal phenotype when they transplanted into adult or immature host tissues of mice, which seemed not to be the result of lacking environmental instructive signals^[24]. It is speculated that the timing and capacity for NSC migration are directly related to their genotype, particularly to the body size. Thus the human neurospheres transplanting into the rodent brain may migrate a very long distance, but in homografts, this migration would represent a small distance^[27].

The reported migration patterns of the grafted NSCs included: (i) target-directed migration along the rostral migratory stream to the olfactory bulb or the granular cell layer after transplanted into the SVZ or the hippocampus, respectively; (ii) non-directed migration in the grey matter in striatum and hippocampus; and (iii) extensive migration within white matter tracts^[28]. We observed that the grafted ESC-derived NPCs changed their migration direction and toward the A β injection site. Most of the differentiated migrating NPCs were obviously located at the same side of A β -deposition, implying the target migration of transplanted NPCs. Thus raising the question: What guided the migration direction of grafted NPCs? It maybe like the migration of lymphocytes that depends on the action of chemokines, considering some chemoattractant such as monocyte chemoattractant protein-1 (MCP-1) is also expressed by astrocytes, microglia and neurons in the CNS^[29]. Recently, researches indicated that both the host environmental cues and the intrinsic properties of the grafted NPCs directed the migration of NSCs^[30,31]. The molecules involved in neuronal migration include soluble factors and cell-bound molecules, and the range is continuously expanding^[32].

The present study showed that most transplanted cells in the injection site remained undifferentiated at the early stage. The reason may be that host inflammatory and immunologic reactions changed the original host neurogenic microenvironment at the transplantation site. As far as those grafts migrated away from the transplantation site, the differentiation rate of the engrafted cells was accordingly increased as time went on. It suggests that NPCs represent their neural plasticity in adult brain tissue only when they migrate into the target areas. The differentiation rate kept rising as the migration distance extending. At 16 weeks post-transplantation, both the glial differentiation rate and the neuronal differentiation rate increased, though the result was comparatively lower than that of Muraoka's

report^[22]. The differentiation fate of grafted cells depends on the brain region which they are transplanted to. For instance, when being transplanted into the hippocampus (which is one of the neurogenic regions and is suitable for the neuronal differentiation), the NPCs will primarily differentiate into interneurons in both the dentate gyrus and the CA1-3 regions; and occasionally differentiate into granule-like neurons.

The neurotoxic A β deposits induced neuron loss is characteristic of AD. Did the remaining A β affect the viability or differentiation potential of the transplanted NPCs in rats? There are contradictory evidences concerning the effect of A β . It is reported that A β peptide affects the viability but not the differentiation of the embryonic and adult hippocampal progenitor cells of rats^[33]. This result would challenge the necessity of selecting a NSC line as a candidate for transplantation according to its response to A β *in vitro*.

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胚胎干细胞来源的神经前体细胞移植 β -淀粉样蛋白损伤大鼠海马后的靶向迁移与分化

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摘要: **目的** 观察小鼠胚胎干细胞来源的神经前体细胞移植 β -淀粉样蛋白(amyloid β peptide, A β)损伤大鼠海马后的靶向迁移及在体分化。**方法** 采用无血清培养法将表达绿色荧光蛋白(enhanced green fluorescent protein, EGFP)的小鼠胚胎干细胞定向诱导为神经前体细胞, 移植至A β_{1-40} 单侧损伤的大鼠海马; 免疫荧光观察移植细胞的迁移距离、迁移方向与分化情况; 对移植细胞的平均迁移距离与平均分化率作相关性分析。**结果** 胚胎干细胞经改良的无血清培养法生长可分化为nestin阳性神经前体细胞。移植后第16周, 神经前体细胞平均迁移距离比第4周增长了约5倍。移植细胞中, 胶质纤维酸性蛋白(glial fibrillary acidic protein, GFAP)阳性细胞的平均分化率从(30.41 $\pm$ 1.45)%增长到(49.25 $\pm$ 1.23)%; 神经丝蛋白200(neurofilament 200, NF200)细胞的平均分化率从(16.68 $\pm$ 0.95)%增长到(27.94 $\pm$ 1.21)%; 靶向迁移至A β 斑块同侧的GFAP阳性细胞的比例从60.2%增长到81.3%, 靶向迁移至A β 斑块的NF200阳性细胞的比例从61.3%增至84.1%。相关性分析结果显示平均迁移距离与神经元分化率之间存在显著线性相关($r=0.991$), 与胶质细胞分化率之间也存在显著线性相关($r=0.953$)。**结论** 胚胎干细胞来源的神经前体细胞移植A β 损伤模型大鼠海马后能够靶向迁移至A β 损伤区并分化为胶质细胞和神经元。**关键词:** 胚胎干细胞; β -淀粉样蛋白; 细胞移植; 分化; 迁移; 大鼠