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Directional induction of dopaminergic neurons from neural stem cells using substantia nigra homogenates and basic fibroblast growth factor☆

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Abstract

To date, complex components of available reagents have been used for directional induction of neural stem cells into dopaminergic neurons, resulting in a poor ability to repeat experiments. This study sought to investigate whether a homogenate of the substantia nigra of adult rats and/or basic fibroblast growth factor could directionally induce neural stem cells derived from the subventricular zone of embryonic rats to differentiate into dopaminergic neurons. Tyrosine hydroxylase-positive cells were observed exclusively after induction with the homogenate supernatant of the substantia nigra from adult rats and basic fibroblast growth factor for 48 hours *in vitro*. However, in the groups treated with homogenate supernatant or basic fibroblast growth factor alone, tyrosine hydroxylase expression was not observed. Moreover, the content of dopamine in the culture medium of subventricular zone neurons was significantly increased at 48 hours after induction with the homogenate supernatant of the substantia nigra from adult rats and basic fibroblast growth factor. Experimental findings indicate that the homogenate supernatant of the substantia nigra from adult rats and basic fibroblast growth factor could directionally induce neural stem cells derived from the subventricular zone of embryonic rats to differentiate into dopaminergic neurons in the substantia nigra with the ability to secrete dopamine.

Key Words: directional induction *in vitro*; homogenate of substantia nigra; basic fibroblast growth factor; subventricular zone; neural stem cells; dopaminergic neurons

Abbreviations: NSC, neural stem cells; SVZ, subventricular zone; PD, Parkinson's disease; HSN, homogenate of substantia nigra; bFGF, basic fibroblast growth factor

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INTRODUCTION

Currently, neural stem cells (NSCs), with their capacity for self-renewal and multipotency, are a promising cell resource commonly employed in therapies for neurodegenerative diseases. It is well known that if NSCs are induced to directionally differentiate into neurons with specific functions prior to transplantation *in vitro*, they will play a critical role in the improvement of neurodegenerative diseases, such as Parkinson's disease (PD)[1]. An increasing number of studies[2-6] have demonstrated that ectogenic environmental factors, such as some growth factors, cytokines and hormones, could influence and modulate the differentiation of NSC populations. Among these factors, basic fibroblast growth factor (bFGF) and epidermal growth factor have been well documented to promote the differentiation of NSCs into neurons and glial cells[7-10] and these are commonly used in experimental research of NSC induction. Therefore, we sought an effective therapeutic strategy for PD based on NSC directional induction.

To our knowledge, there have been few reports of directional induction differentiation of NSCs into dopaminergic neurons *in vitro*[11]. A previous study[12] demonstrated that neurons derived from the subventricular zone (SVZ) in embryonic rats were mostly nestin-positive NSCs, thus attracting our interest in the plasticity and directional induction of NSCs. However, few studies have investigated NSC therapy for PD prior to directional induction into dopaminergic neurons *in vitro*.

In the present study, we attempted to investigate whether the NSCs derived from SVZ neurons in embryonic rats can differentiate into substantia nigral dopaminergic neurons following induction with a homogenate of substantia nigra (HSN) and/or bFGF.

The application of NSC transplantation for the treatment of neurodegenerative diseases is a current hotspot of neuroscience research. However, increasing evidence[13] suggests that NSCs

that are directionally induced toward specific neurons prior to transplantation possess greater efficacy. Many studies to date[2-10] have demonstrated that the proliferation and differentiation of plastic NSCs are modulated by various extrinsic factors, such as growth factors, cytokines and hormones, but many of these are only involved in the induced differentiation of NSCs into neurons and glial cells.

Loss of dopaminergic neurons in the substantial nigra is a predominant manifestation of PD. Therefore, it is important to deal with the issue of how to direct NSCs to differentiate into dopaminergic neurons. In recent similar studies[14-15], retinoic acid has been commonly employed alone or in combination with other factors for stem cell induction into specific functional neurons, including dopaminergic neurons. However, the complexity of the inducing reagent has led to a poor ability to repeat the findings of experiments, and has delayed the development and application of cellular induction techniques required for central nervous system disease therapy. bFGF is commonly used to induce stem cells, including NSCs into neurons and glial cells[15-17]. However, whether bFGF can direct NSCs to differentiate into dopaminergic neurons, and whether the microenvironment of HSN can facilitate this induction process, still remains unclear.

To resolve the above issues, in the present study, we used HSN derived from adult rat and/or bFGF to induce NSCs derived from the SVZ of embryonic rats to differentiate into dopaminergic neurons. Immunocytochemistry and fluorospectrophotometry were used to observe tyrosine hydroxylase (TH) expression in differentiated cells and their secretion of dopamine.

RESULTS

Immunocytochemical identification of the SVZ neurons of embryonic rats before induction

Immunocytochemistry for nestin showed that, primary cultured SVZ neurons from embryonic rats exhibited immunopositivity as brown cells, and were distributed in a lamellar fashion. Nuclear staining was much stronger than cytoplasmic staining (Figure 1A). After counterstaining with hematoxylin, brown nestin-positive cells and blue nestin-negative cells were visible under the same visual field (Figure 1B). These findings suggested that the SVZ neurons derived from the embryonic rats were NSCs.

Immunohistochemical identification of dopaminergic neurons before induction

The neurons derived from the dorsolateral midbrains of adult rats showed brown immunopositivity for TH with a lamellar distribution by immunocytochemistry (Figure 2A). When counterstained with hematoxylin, brown TH-positive cells were seen to be distributed among blue negative cells in the same visual field (Figure 2B). The detection of TH confirmed these cells to be dopaminergic neurons.

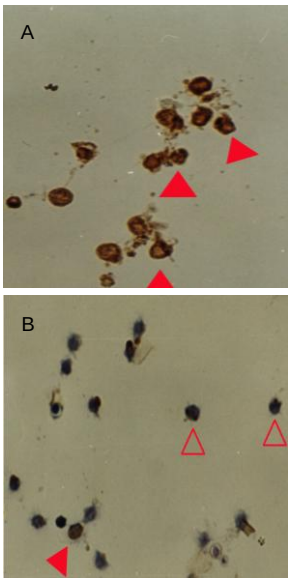

Figure 1 Nestin-positive cells derived from subventricular zone neurons obtained from rat embryos after primary culture for 48 hours (immunocytochemistry, light microscopy, × 400).

(A) Brown patchy neurons in the rat embryonic subventricular zone labeled using an anti-nestin antibody (▲).

(B) Brown nestin-positive neurons in the rat embryonic subventricular zone (▲) among the blue immunonegative cells (△) following immunocytochemistry and hematoxylin staining.

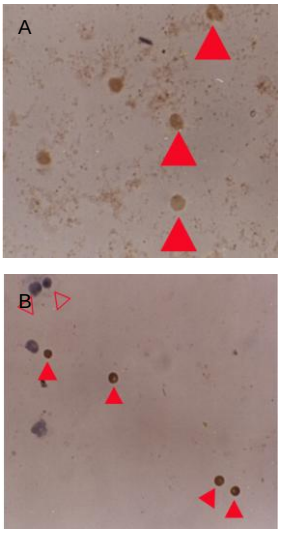

Figure 2 Tyrosine hydroxylase-positive cells derived from the substantia nigra of adult rats after primary culture for 48 hours (immunocytochemistry, light microscopy, × 400).

(A) Brown patchy tyrosine hydroxylase-positive neurons (▲).

(B) Brown tyrosine hydroxylase-positive neurons (▲) among negative cells (△) following immunocytochemistry and hematoxylin staining.

Directional induction of SVZ neurons

Immunocytochemistry showed that lamellar brown TH-immunopositive cells were exclusively observed in the HSN + bFGF group. When counterstained with hematoxylin, brown TH-positive cells distributed among blue negative cells were found in the same visual field (Figure 3A). The immunoreactivity was mainly located in the cytoplasm of positive cells, while their nuclei were stained blue (Figures 3B, C). However, no brown TH-immunopositive cells were observed in tissue induced with HSN or bFGF induced alone, or receiving no treatment.

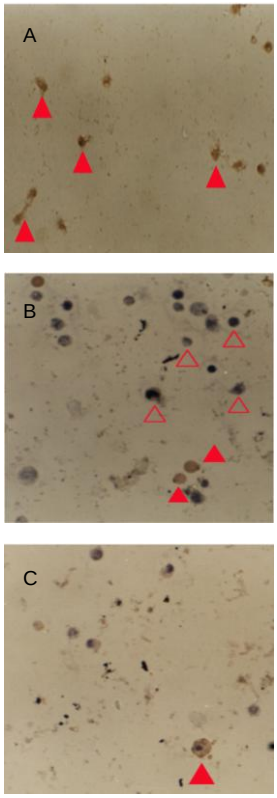

Figure 3 Neural stem cells differentiated into dopaminergic neurons (immunocytochemistry, light microscopy, × 400).

(A) Brown tyrosine hydroxylase-positive neurons (▲) primary cultured and induced by homogenate of substantia nigra + basic fibroblast growth factor for 48 hours from neural stem cells detected by immunocytochemistry.

(B, C) In tyrosine hydroxylase-positive neurons (▲), the cytoplasm was positively stained and the nuclei were negative (B: △; C: ▲); anti-tyrosine hydroxylase immunocytochemistry followed by hematoxylin staining.

Dopamine content assay in the culture medium

Fluorospectrophotometry was used to assay dopamine content. The fluorescence intensity in the culture medium of each experimental group (10 culture wells) and the change in dopamine content in each group at 0, 24 and 48 hours after directional induction with HSN + bFGF are shown in Figure 4. The mean dopamine contents (ng/mL) in the culture medium of SVZ neurons in embryonic rats at 0, 24 and 48 hours after directional induction were 101.33 ± 88.43, 71.77 ± 17.46, and 228.30 ± 50.10, respectively. Statistical analysis showed that the dopamine content after induction for 48 hours was significantly increased ($P < 0.01$).

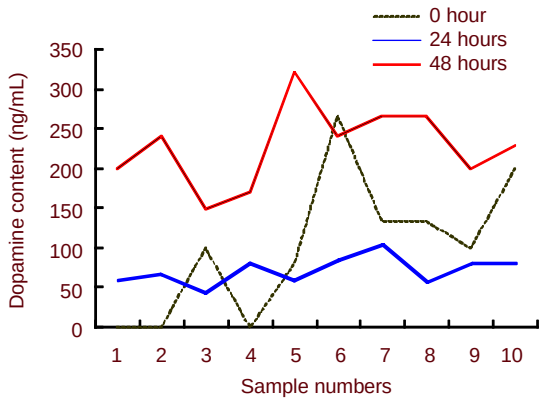

Figure 4 Change in dopamine content in the culture medium of subventricular zone neurons in embryonic rats at 0, 24 and 48 hours after directional induction.

DISCUSSION

Increasing evidence[2-3] suggests that bFGF has the potential to induce different NSC populations derived from different tissues of rats to proliferate, self-renew and differentiate into neurons, astrocytes and oligodendrocytes *in vitro*. Palmer *et al*[18] found that some NSCs that ought to have developed into glial cells when exposed to fibroblast growth factor-2 could in fact be induced to differentiate into neurons. Shah *et al*[19] found that transforming growth factor-β could improve the differentiation of neural crest stem cells into smooth muscle cells *in vivo*. Shetty *et al*[3] demonstrated that brain-derived nerve growth factor promoted the differentiation of NSCs in the hippocampus, cerebral cortex, cerebellum, midbrain and corpus striatum derived from fetal mice into different types of neurons. Different growth factors can induce NSCs under certain conditions, either solely or combined with other ectogenic factors to direct differentiation into specific functional neurons in the central nervous system. For instance, Bjornson *et al*[20] engrafted labeled NSCs into myelosuppressive mice irradiated by X-ray and found that they produced a series of hemocytes, such as bone marrow cells, lymphocytes and pristine hematopoietic cells in the host hemopoietic system. These findings suggested that NSCs have much more extensive plasticity for differentiation than was previously thought. In the present study, based on the features of bFGF, we selected bFGF, a growth factor that can induce NSCs *in vitro*, as one of the inducing reagents. However, only in the HSN + bFGF group did the NSCs derived from the SVZ of embryonic cells successfully differentiate into dopaminergic neurons, indicating that a component of

the HSN may provide NSCs in the SVZ with a favorable microenvironment *in vitro*, allowing them to differentiate into dopaminergic neurons. The inductive methods in this study represent a new attempt to direct NSCs in the SVZ, cells possessing multipotent capacity and distinct plasticity, to differentiate. Importantly, HSN and bFGF identically contributed to the process of directional induction.

Notably, our results using qualitative (immunocytochemistry) and quantitative (fluorospectrophotometry) methods showed that the induced dopaminergic neurons were successfully derived from SVZ neurons of embryonic rats. We also found that the dopamine content at 48 hours after induction reached a relatively high level, suggesting that, after induction, the SVZ neurons not only directionally differentiate into substantia nigral neurons, but also have the ability to secrete dopamine. Dopamine secretion peaked between 24 hours and 48 hours after induction. The timing for induction of SVZ neurons at the primary stage (primary cultured for 3–5 days) was selected to precede the time when the NSCs would differentiate into other types of cells. Furthermore, immunocytochemistry of NSCs in SVZ showed most of them to be nestin immunopositive after primary culture for 3–5 days. Because nestin is a marker of undifferentiated NSCs[20], we determined that, when primary cultured for 3–5 days, most of the NSCs in the SVZ, which were 50–60% confluent, had not yet differentiated. On the other hand, when primary cultured for 3–5 days, NSCs were in their exponential proliferation stage, which is the optimal timing for all kinds of study on cells. Thus, we concluded that 3–5 days after primary culture was the optimal timing for directional induction of these SVZ neurons. To our knowledge, there have been few reports of directional induction of NSCs to differentiate into dopaminergic neurons *in vitro*.

In summary, in this study, we found that HSN + bFGF can effectively and directionally induce NSCs to differentiate into dopaminergic neurons with the ability to secrete dopamine. The present study will shed new light on future therapies for PD involving NSC transplantation after directional induction *in vitro.* In future, given the successful directionally differentiation of NSCs into dopaminergic neurons, extensive studies of NSC transplantation after directional induction are required so that new and promising strategies for clinical PD treatment using NSC transplantation can be realized.

MATERIALS AND METHODS

Design

A neurobiology and cellular experiment.

Time and setting

The experiment was performed at the Institute of Oncology in the First Affiliated Hospital of Kunming Medical College, China, from July 1999 to March 2001.

Materials

A total of 20 Sprague-Dawley rats, at gestational days 12–16, weighing 370–480 g, were used in NSC extraction and identification experiments. A total of 30 Sprague-Dawley rats, aged 10 weeks, weighing 220–300 g, irrespective of gender, were used to prepare HSN. All rats were provided by the Experimental Animals Center of Kunming Medical College, China (certificate No. SCXK (Dian) 2005-0007). The treatment and care of animals was in accordance with the *Administrative Regulations on Medical Institution*, formulated by the State Council of China[21].

Methods

Culture of SVZ neurons of embryonic rats in vitro

Sprague-Dawley rats at 12–16 days gestation were anesthetized intraperitoneally with thiopental sodium (40 mg/kg). The peritoneum was opened and fetal rats were taken out. With the help of an anatomical microscope (Nikon, Tokyo, Japan), brain tissues were isolated from the cranial cavities of embryonic rats. After removal of the forebrain, midbrain and brain stem, the SVZ tissues, ventral to the cerebral cortex, were harvested[22]. SVZ tissues were put in D-Hank's solution and poached for 10–15 minutes, followed by repeated trituration and digestion using 0.02% ethylenediamine tetraacetic acid and 0.25% trypsin (1:1) for 30 minutes. Following filtration through a 40 μm sterile filter membrane, bovine fetal serum was added to stop digestion. Filtrates were centrifuged at 1 000 r/min for 5 minutes and serum culture medium was added. Then, a single cell suspension was formed after blowing repeatedly with a Pasteur pipette. Last, the single cellular suspension was seeded onto 6-well culture plates (on which cover glasses had previously been placed) and a 75-cm^2 culture flask at a density of 5–6 ×10^6 cells/mL. Cells were incubated in the presence of 5% CO_2, under saturated humidity at 37°C and incubated for 24 hours. After that, the serum culture medium was replaced with a serum-free medium. Continuous culture lasted for 3–7 days. Every 2 days, 5 mL of fresh serum-free culture medium was added to the culture system[23].

NSC identification from SVZ neurons by immunocytochemistry

The cover glasses in the 6-well culture plates were removed and fixed with 4% paraformaldehyde and 0.1 M phosphate buffer (pH 7.4) for 4–6 hours. Then, immunocytochemistry using a nestin antibody (Becton Dickinson, Franklin Lakes, NJ, USA) and the SP method[24] were performed to identify the SVZ neurons in embryonic rats as NSCs.

Harvest, identification and homogenization of substantia nigral tissues

Adult Sprague-Dawley rats were anaesthetized with thiopental sodium (40 mg/kg) intraperitoneally. The cranial cavity was opened and the brain tissues in the dorsolateral midbrain[25] were harvested. The brain tissues were poached in D-Hank's solution for 10–15 minutes, and then sheared, digested, filtrated thoroughly and cultured. In brief, Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) + 10% fetal

bovine serum was used as a culture medium. The single cell suspension was seeded into 6-well culture plates with cover glasses in each well at a density of 6×10^6 cells/mL. After culture for 48 hours, the cover glasses were removed and immunocytochemistry using a TH antibody (1:8 000; Sigma, St. Louis, MO, USA) and the SP method[24] was performed to confirm the neurons derived from the dorsolateral midbrain as dopaminergic neurons.

Dorsolateral midbrain tissues derived from adult rats were poached in D-Hank's solution for 10–15 minutes, and completely homogenized in Dulbecco's modified Eagle's medium using an electrokinetic homogenate apparatus (Jiang Xing Instrument Co., Ltd., Hangzhou City, Zhejiang Province, China). Thereafter, the homogenate solution was centrifuged at 2 000 r/min for 10 minutes. The obtained supernatants were ready to use in the NSC-induction process.

Induced differentiation of NSCs

Meanwhile, 10 μg bFGF (Gibco) was dissolved in 5 mL of distilled water. A microporous membrane was used to filter and sterilize the bFGF solution, which was placed in Eppendorf tubes and served as another kind of inducing solution for subsequent experiments.

Cover glasses containing SVZ neurons (primary cultured for 3–5 days) in 6-well culture plates were divided into four groups randomly: an HSN group, in which supernatant of HSN was used to induce SVZ neurons; a bFGF group, in which bFGF was used to induce SVZ neurons; an HSN + bFGF group, in which HSN + bFGF were used simultaneously; and a blank control group, with no treatment. Each cover glass commonly achieved 50–60% confluent neuronal growth. In each group, two cover glasses were randomly selected to undergo induction. The volume of HSN added to each well was 1 mL. The concentration of bFGF induction solution was 20 ng/mL[26], and 0.5 mL of bFGF was added to each well. Culture plates were placed in an incubator containing 5% CO_2 at 37°C for 48 hours, for induction.

Immunocytochemistry for SVZ neurons after induction

Cover glasses were removed from culture plates 48 hours after induction, and fixed with 4% paraformaldehyde and 0.1 M phosphate buffer (pH 7.4). Then, immunocytochemistry[27] with a monoclonal goat-anti-rat TH antibody (1:8 000; Sigma) was performed to examine whether the induced SVZ neurons differentiated into dopaminergic neurons. Diaminobenzidine was used for coloration. A light microscope (Olympus, Tokyo, Japan) was used to observe immunostaining.

Measurement of dopamine content in culture medium of SVZ neurons after induction

Because TH-immunopositive cells were only observed in the HSN + bFGF group in the induction experiment, we only used HSN + bFGF as the inducer in this experiment. Briefly, a total of 10 culture wells in which 50–60% confluent SVZ neurons grew on the cover glass, were selected for induction. One milliliter of HSN and 0.5 mL of bFGF (20 ng/mL) were added to each well for induction. At time points of 0, 24, 48 hours after induction, 0.5 mL of culture medium was taken from selected holes under sterile conditions and placed in Eppendorf tubes. Fluorospectrophotometry (Toshiba, Tokyo, Japan)[28] was used to assay the dopamine content in the culture medium of each well. Culture and induction conditions were the same as those described for SVZ neurons.

Statistical analysis

Data were analyzed by paired *t*-tests using SPSS 11.5 software (SPSS, Chicago, IL, USA). Data are expressed as mean ± SD. A level of $P < 0.05$ was considered to represent statistical significance.

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Author contributions: Jintao Li had full access to the study concept and design, was responsible for the data analysis, statistical analysis and funding, and drafted the manuscript. Yiliu Ma and Zhongtang Feng were responsible for the theoretical and experimental instructions. Yiliu Ma and Tinghua Wang supervised the manuscript. Qi Yan performed the animal experiments.

Conflicts of interest: None declared.

Ethical approval: The experimental protocols were approved by the Animal Ethics Committee of Kunming Medical College in China.

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