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Distribution and localization of fibroblast growth factor-8 in rat brain and nerve cells during neural stem/progenitor cell differentiation☆

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Abstract
The present study explored the distribution and localization of fibroblast growth factor-8 and its potential receptor, fibroblast growth factor receptor-3, in adult rat brain *in vivo* and in nerve cells during differentiation of neural stem/progenitor cells *in vitro*. Immunohistochemistry was used to examine the distribution of fibroblast growth factor-8 in adult rat brain *in vivo*. Localization of fibroblast growth factor-8 and fibroblast growth factor receptor-3 in cells during neural stem/progenitor cell differentiation *in vitro* was detected by immunofluorescence. Flow cytometry and immunofluorescence were used to evaluate the effect of an anti-fibroblast growth factor-8 antibody on neural stem/progenitor cell differentiation and expansion *in vitro*. Results from this study confirmed that fibroblast growth factor-8 was mainly distributed in adult midbrain, namely the substantia nigra, compact part, dorsal tier, substantia nigra and reticular part, but was not detected in the forebrain comprising the caudate putamen and striatum. Unusual results were obtained in retrosplenial locations of adult rat brain. We found that fibroblast growth factor-8 and fibroblast growth factor receptor-3 were distributed on the cell membrane and in the cytoplasm of nerve cells using immunohistochemistry and immunofluorescence analyses. We considered that the distribution of fibroblast growth factor-8 and fibroblast growth factor receptor-3 in neural cells corresponded to the characteristics of fibroblast growth factor-8, a secretory factor. Addition of an anti-fibroblast growth factor-8 antibody to cultures significantly affected the rate of expansion and differentiation of neural stem/progenitor cells. In contrast, addition of recombinant fibroblast growth factor-8 to differentiation medium promoted neural stem/progenitor cell differentiation and increased the final yields of dopaminergic neurons and total neurons. Our study may help delineate the important roles of fibroblast growth factor-8 in brain activities and neural stem/progenitor cell differentiation.

Key Words
fibroblast growth factor-8; fibroblast growth factor receptor-3; neural stem/progenitor cell differentiation; dopaminergic neurons; midbrain; neural regeneration

Research highlights
(1) Fibroblast growth factor-8 and one of its receptor, fibroblast growth factor receptor-3, localized on the plasma membrane and in the cytoplasm of cells differentiated from neural stem/progenitor cells. (2) Fibroblast growth factor-8 is distributed in the substantia nigra, compact part, dorsal tier, substantia nigra and reticular areas of the midbrain, but not in the caudate putamen and striatum of the forebrain, and an unusual distribution is observed in retrosplenial locations of adult rat brain. (3) An anti-fibroblast growth factor-8 antibody or recombinant fibroblast growth factor-8 significantly impairs or promotes *in vitro* expansion and differentiation of neural stem/progenitor cells, respectively.

Abbreviations

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SNCD, substantia nigra, compact part, dorsal tier; SNR, substantia nigra, reticular part; Cpu, caudate putamen, striatum; V1B, primary visual cortex, binocular area; V1M, primary visual cortex, monocular area; V2MM, secondary visual cortex, mediomedial area

INTRODUCTION

The fibroblast growth factor family consists of 23 members[1]. Fibroblast growth factors exert their functions in organisms and pathological processes *via* binding to their receptors[2]. Fibroblast growth factor receptors are distributed abundantly among tissues and cells[2-4]. Many studies have demonstrated that fibroblast growth factors play vital roles in nervous system development and regeneration[2-5]. Ford-Perriss *et al* [5] discovered that more than 10 types of fibroblast growth factor and fibroblast growth factor receptors 1–4 are widely expressed during nervous system development. Fibroblast growth factors and fibroblast growth factor receptors can affect the occurrence and renewal of neurons and glial cells, as well as cell proliferation and outgrowth in important brain areas such as the cerebral cortex, cerebellar cortex, forebrain and midbrain[5-6]. Signal transduction of fibroblast growth factor and fibroblast growth factor receptor binding is also required for the formation and growth of many embryonic organs[7-8]. In the present study, we explored the distribution of fibroblast growth factor-8 and fibroblast growth factor receptor-3 in adult brain *in vivo*, and in cells during neural stem/progenitor cell differentiation *in vitro*[9-11]. Our study may help to delineate the roles of fibroblast growth factor-8 in central nervous system activities and neural stem/progenitor cell differentiation.

RESULTS

Distribution of fibroblast growth factor-8 in adult rat brain

Fibroblast growth factor-8 was widely distributed in the adult brain, particularly in the substantia nigra, compact part, dorsal tier (SNCD) and substantia nigra, reticular part (SNR) of the midbrain. Immunohistochemistry results are shown in Figure 1.

Figure 1A shows sections of the caudate putamen, striatum (Cpu) of the rat brain, which did not stain for fibroblast growth factor-8. Figure 1B shows sections of the SNCD and SNR, which were significantly stained positive for fibroblast growth factor-8. Fibroblast growth factor-8 localized to the plasma membrane (Figure 1B-i; large black arrows) and cytoplasm (Figure 1B-i; red arrows) of positively stained cells.

Figure 1C shows sections of the primary visual cortex, binocular area (V1B), primary visual cortex, monocular area (V1M), secondary visual cortex, mediomedial area (V2MM), rubrospinal tract and the retrosplenial locations (retrosplenial agranular cortex; retrosplenial granular a cortex; retrosplenial granular b cortex) of the brain, which showed an unusual distribution pattern of fibroblast growth factor-8.

Localization of fibroblast growth factor-8 and fibroblast growth factor receptor-3 in cells during neural stem/progenitor cell differentiation *in vitro*

Fibroblast growth factor-8 and fibroblast growth factor receptor-3 were colocalized on the plasma membrane and in the cytoplasm of differentiated neural stem/progenitor cells (Figure 2).

Non-permeable and permeable treatment groups consisted of treatment with or without Triton X-100 to permeabilize the plasma membrane. Fibroblast growth factor-8 was detected in the cytoplasm and on the plasma membrane of cells. Immunofluorescence results are shown in Figure 2. The control groups (Figures 2A, E) showed no fluorescence. In non-permeable treatment groups (Figures 2B, F), fibroblast growth factor-8 and fibroblast growth factor receptor-3 were detected on the plasma membrane (Figures 2B, F; large arrows), indicating that fibroblast growth factor-8 and fibroblast growth factor receptor-3 colocalized on the plasma membrane.

In permeable treatment groups (Figures 2C, D, G, H), several cells showed significant immunostaining for fibroblast growth factor-8 and fibroblast growth factor receptor-3 in the cytoplasm or on the cell surface (Figures 2C, D, G, H).

Effects of an anti-fibroblast growth factor-8 antibody on neural stem/progenitor cell expansion and differentiation

Neural stem/progenitor cell proliferation and differentiation were significantly impaired by attenuation of fibroblast growth factor-8. Based on morphological observation under a microscope and flow cytometric analysis, we found that the ratios of undifferentiated neural stem/progenitor cells (nestin$^+$ cells) to total cells in the anti-fibroblast growth factor-8 treatment group were significantly lower than those in control groups at 3, 10 and 20 days of differentiation ($P < 0.05$; Figure 3, Table 1).

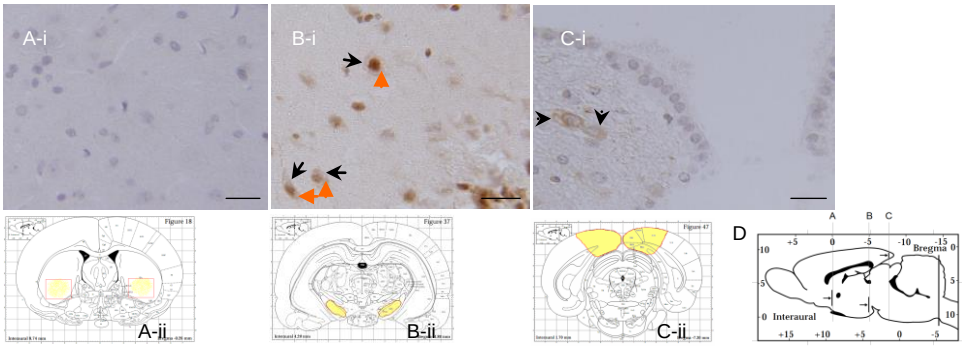


Figure 1 Distribution pattern of fibroblast growth factor-8 (FGF-8) in adult rat brain, as detected by immunohistochemistry.

Observed under a Leica DMIRE2 inverted microscope. Scale bars, A-i, B-i, C-i: 50 μm. *n* = 3. The localization of fibroblast growth factor-8 in the brain is shown in A-ii, B-ii, C-ii and D. (A) Negative (−); (B) positive (+); C: Unusual (±). Positive staining of fibroblast growth factor-8 on the plasma membrane and in the cytoplasm is indicated by black and red arrows, respectively.

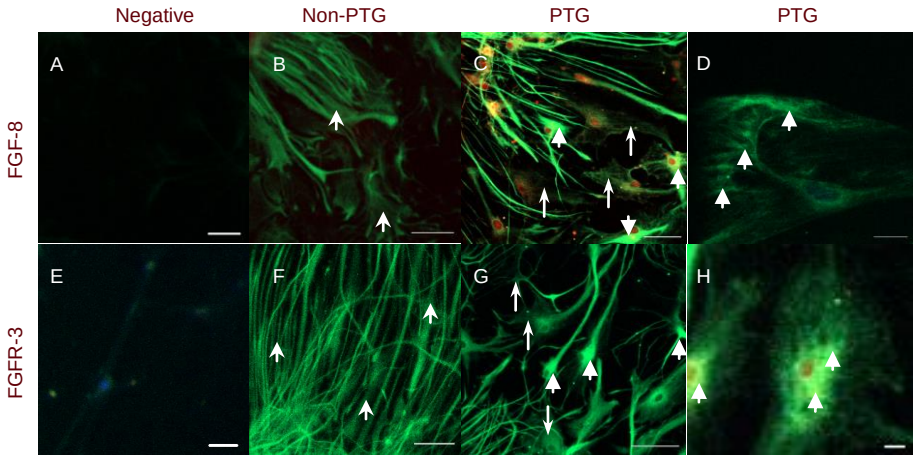


Figure 2 Immunofluorescence analyses of fibroblast growth factor-8 (FGF-8) and fibroblast growth factor receptor-3 (FGFR-3) localization in cells during neural stem/progenitor cells (NSCs/NPCs) differentiation (confocal microscope).

(A–D) FGF-8$^+$ (green, FITC); (E–H) FGFR-3$^+$ (green, FITC). Green: FITC, FGF-8$^+$ or FGFR-3$^+$; red: TRITC, nestin$^+$; blue: DAPI, nuclei; multicolor: merged.

(C, D, G, H) Triangle arrows indicate positive staining in the cytoplasm in permeable treatment groups. Thin arrows indicate negative staining. (B, F) Large arrows indicate positive staining on the plasma membranes of differentiated cells in NSCs/NPCs in non-permeable treatment groups. Scale bars: (A, E) 50 μm; (B, C, F, G) 100 μm; (D, H) 20 μm. *n* = 4. Non-PTG: non-permeable treatment group; PTG: permeable treatment group. FITC: Fluorescein isothiocyanate; TRITC: tetraethylrhodamine isothiocyanate.

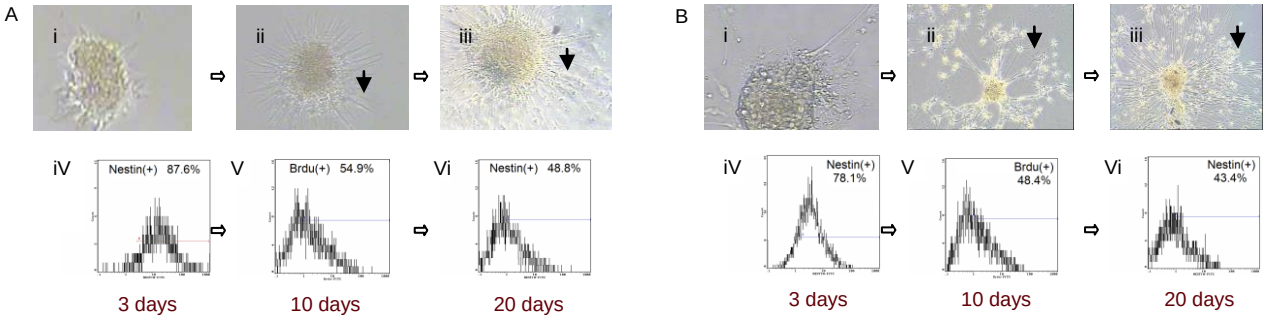


Figure 3 Effects of an anti-fibroblast growth factor-8 (FGF-8) antibody on neural stem/progenitor cell (NSC/NPC) differentiation and expansion.

(A, B) i–iii: differentiation statuses in the 3rd, 10th and 20th day stages by light microscope observation; iv–vi: the corresponding detection results of percentages of nestin$^+$ cells *vs*. total. Observed under a Leica inverted microscope and evaluated by cells positive for nestin by immunofluorescence and flow cytometric analyses. (A) Treatment group: the effects of adding anti-FGF-8 antibodies to the culture medium at 10 and 20 days during NSC/NPC differentiation stages. (B) Control group: the results of normal NSC/NPC differentiation under differentiation conditions. Scale bars: 100 μm.

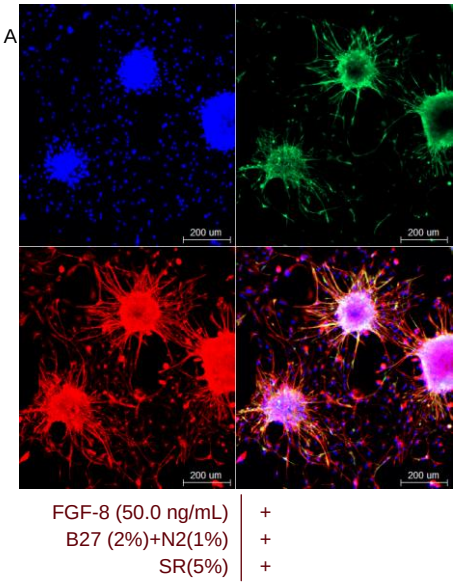
Table 1 Comparison of the ratios of undifferentiated cells (nestin$^+$) to total cells between treatment and control groups at 3, 10, 20 days

Group	3 days	10 days	20 days
Treatment	81.7±11.8[a]	58.1±9.3[a]	50.4±6.8[a]
Control	74.4±15.6	41.9±16.4	31.2±17.6

Nestin$^+$ cells were analyzed by immunofluorescence and flow cytometry[9-10]. [a]$P < 0.05$, *vs*. control group (Duncan multiple comparison test). Measurement data are presented as mean ± SD (n = 4).

Neural stem/progenitor cell differentiation by adding fibroblast growth factor-8 to differentiation medium

We explored the effects of inducing the differentiation of neural stem/progenitor cells into neurons, particularly dopaminergic neurons, by adding fibroblast growth factor-8 to the differentiation medium. As shown in Figure 4 and Table 2, obviously different neurons and a higher percentage of total neurons and dopaminergic neurons were detected after adding 50 ng/mL recombinant fibroblast growth factor-8 to the differentiation medium ($P < 0.05$).



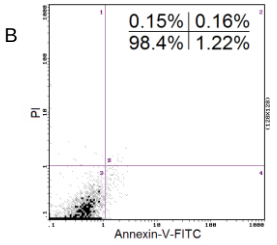


Figure 4 Promotion of neural stem/progenitor cell differentiation into neurons including dopaminergic neurons by addition of fibroblast growth factor-8 (FGF-8) (confocal microscope).

(A) Blue (DAPI): nuclei; green (FITC): TH$^+$; red (TRITC): NSE$^+$; multicolor: merged. Scale bars: 200 μm.

(B) An annexin-V FITC & propidium iodide (PI) apoptosis assay at 20 days of differentiation showed the count of annexin-V-positive apoptotic cells, and the count of PI-stained necrotic cells. TH: tyrosine hydroxylase; NSE: neuron specific enolase; SR: serum replacement.

Table 2 FGF8-induced neuronal differentiation of neural stem/progenitor cells at 20 days

	% Nestin$^+$/total	% NSE$^+$/total	% TH$^+$/total	% TH$^+$/NSE$^+$	% apoptotic cells/total
Control [B27(2%)+N2(1%)] + [SR(5%)]	34.6±13.5	8.9±3.8	1.0±0.5	11.6±6.1	5.9±4.2
Inducement [B27(2%)+N2(1%)] + [SR(5%)]+[FGF-8 (50.0 ng/mL)]	26.5±14.2[a]	27.8±14.2[a]	8.6±4.3[a]	30.8±15.1[a]	1.2±0.7[a]

Immunostaining and flow cytometry were used to evaluate the percentages of various target cells[12-13]. [a]$P < 0.05$, *vs*. control group (Duncan multiple comparison test). Measurement data are presented as mean ± SD (n = 4). FGF-8: Fibroblast growth factor-8; NSE: neuron specific enolase; TH: tyrosine hydroxylase; SR: serum replacement.

DISCUSSION

Fibroblast growth factor-8 is distributed in adult midbrain

Immunohistochemistry revealed significant positive staining for fibroblast growth factor-8 in the SNCD and SNR of adult rat brain. A positive or unusual distribution pattern was detected in V1B, V1M, V2MM, the rubrospinal tract and retrosplenial, which is consistent with previous studies[14-20]. However, in the Cpu, fibroblast growth factor-8 was not detected. Many studies have reported that fibroblast growth factor-8 is required for the formation and patterning of forebrain components[20-23].

Fibroblast growth factor-8 was significantly involved in neural stem/progenitor cell differentiation, which is consistent with previous studies[24-25]. Thus, a wide distribution of fibroblast growth factor-8 in adult brain may be associated with quiescent neural stem/progenitor cells in the cerebrum that may undergo differentiation in the adult mammalian brain.

Fibroblast growth factor-8 and fibroblast growth factor receptor-3 localize on the plasma membrane and in the cytoplasm

We observed obvious positive staining for fibroblast growth factor-8 and fibroblast growth factor receptor-3 surrounding nuclei. The observed fibroblast growth factor-8 or fibroblast growth factor receptor-3 in individual cells may be synthesized by the cell itself and localized in ribosomes[26-28].

The major aim of this study is to illustrate that fibroblast growth factor-8 is an exocrine factor, in which fibroblast growth factor-8 enters the intercellular fluid, tissue fluid or circulatory system by paracrine or endocrine manners, and exerts its effects on target cells in various regions[29-30]. Results from the present study demonstrated that fibroblast growth factor-8 and fibroblast growth factor receptor-3 are localized to both the plasma membrane and cytoplasm of nerve cells. In the same visual field, fibroblast growth factor-8 and fibroblast growth factor receptor-3 were detected in some cells. Therefore, we believe that a large amount of fibroblast growth factor-8 and fibroblast growth factor receptor-3 are expressed by the cells themselves, rather than obtained from extracellular sites. However, some nerve cells did not show this kind of distribution pattern, in which negative or weakly positive immunoreactions were observed[9, 29-30]. Fibroblast growth factor receptor-3 located on the cell membrane can bind extracellular fibroblast growth factor-8, which induces signal transduction[31-32]. Fibroblast growth factor-8 located on the cell membrane is partially excreted[9, 29-31].

We selected fibroblast growth factor receptor-3 as a representative fibroblast growth factor receptor. We considered that fibroblast growth factor receptor-3 might be the only fibroblast growth factor receptor that participates in neural stem/progenitor cell differentiation. During differentiation, fibroblast growth factor receptor-3 can bind various fibroblast growth factor ligands. Similarly, fibroblast growth factor-8 can bind to all fibroblast growth factor receptors[1, 33-35].

Neutralized or additional fibroblast growth factor-8 affects neural stem/progenitor cell differentiation

We performed an observational study of attenuated fibroblast growth factor-8 concentrations to explore the effects of downregulation of target factors, which is similar to previous studies[36-37]. We added an anti-fibroblast growth factor-8 antibody to differentiation medium for neural stem/progenitor cells. A decreased concentration of fibroblast growth factor-8 induced remarkable changes in the rate of neural stem/progenitor cell differentiation[37-38]. Fibroblast growth factor-8 is tightly correlated with neural stem/progenitor cell differentiation.

In addition, we studied the effect of additional fibroblast growth factor-8 during *in vitro* neural stem/progenitor cell differentiation. Recombinant fibroblast growth factor-8 was added to the differentiation medium of neural stem/progenitor cell cultures. As a result, there was a significant difference in differentiation between the 50 ng/mL fibroblast growth factor-8 group and control group, indicating that fibroblast growth factor-8 affects neural stem/progenitor cell differentiation[24, 39].

The upregulation and downregulation tests also confirmed that fibroblast growth factor-8 is a secretory factor.

Importance of fibroblast growth factor-8 in neural stem/progenitor cell differentiation and other central nervous system actions

The results of attenuation of fibroblast growth factor-8 in the differentiation medium of neural stem/progenitor cells indicate that insufficient synthesis or secretion of fibroblast growth factor-8 in the central nervous system may contribute to growth retardation, physiological degeneration or functional defects. Whether additional fibroblast growth factor-8 can treat growth retardation, physiological degeneration or functional defects of the central nervous system is unknown, but may be clinically applicable based on our study.

In summary, fibroblast growth factor-8 is distributed in adult midbrain. Fibroblast growth factor-8 and fibroblast growth factor receptor-3 are distributed on nerve cell membranes and in the cytoplasm. Addition of an anti-fibroblast growth factor-8 antibody or recombinant fibroblast growth factor-8 to differentiation medium significantly affects neural stem/progenitor cell expansion and differentiation.

MATERIALS AND METHODS

Design

Cytological, comparative, observational study.

Time and setting

Experiments were performed at Taihe Hospital, Hubei University of Medicine, and Guangxi University in China from July 2007 to November 2011.

Materials

Neural stem/progenitor cells were isolated from 6–7 embryonic day (E)14 Sprague-Dawley rat embryos or the fetal hippocampus of 10–12 E14 Kunming mouse embryos[40-41]. Animals were provided by the Laboratory Animal Center of Hubei University of Medicine, China (license No. SCXK (Hubei) 2005-0008). Protocols were conducted in accordance with the *Guidance Suggestions*

for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China[42].

Methods

Immunohistochemical analysis of fibroblast growth factor-8

Paraffin-embedded sections (10 μm) were obtained from the brains of three adult male Sprague-Dawley rats. The cerebral Cpu, SNCD and SNR, V1B, V1M, V2MM, rubrospinal tract and retrosplenial locations were analyzed.

Sections were blocked with 4% goat serum, and then incubated with a rabbit anti-fibroblast growth factor-8 polyclonal antibody (1:30; Bioss, Beijing, China) for 12 hours at 4°C[43], followed by a horseradish peroxidase-conjugated AffiniPure goat anti-rabbit IgG (Thermo Fisher Scientific Inc., Rockford, IL, USA) for 1 hour at room temperature. After rinsing in PBS three times for 10 minutes each, sections were incubated with 3,3-diaminobenzidine tetrahydrochloride for 5–10 minutes[43]. Brain areas were located according to *The Rat Brain in Stereotaxic Coordinates* (George Paxinos and Charles Watson, Academic Press, 1997; San Diego, CA, USA) and a report by Kroon *et al* [44].

Isolation and culture of neural stem/progenitor cells

Neural stem/progenitor cells were cultured in Dulbecco's modified Eagle's medium/F12 (1:1 v/v) containing 2% B27 and 1% N2 supplements, 0.5 mM L-glutamine, 0.5 mM non-essential amino acids, 20 ng/mL basic fibroblast growth factor (Promega, Madison, WI, USA), 50 IU/mL penicillin and 50 μg/mL streptomycin[30, 35, 40]. Neural stem/progenitor cell differentiation medium contained 8% serum replacement (Gibco, Grand Island, NY, USA) and basic fibroblast growth factor was omitted[40, 45-46]. Cells were cultured in flasks and differentiated for 20 days. Half-medium volumes were replaced with fresh medium every 7 days. Cells were observed and evaluated at the 3rd, 10th and 20th days of differentiation.

Immunofluorescence and flow cytometry

Differentiated neural stem/progenitor cells at specific stages were permeabilized with 0.5% Triton X-100 (permeable) or not (non-permeable)[47]. Sections were blocked with 4% goat serum, and then incubated with primary antibodies for 12 hours at 4 °C[47]. After washing, samples were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific Inc.,), or tetraethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (Thermo Fisher Scientific Inc.,) for 1 hour. A confocal microscope (Carl Zeiss GmbH, Jena, Germany) and the confocal analysis system (Carl Zeiss GmbH) were used for analysis[9, 47].

For flow cytometry, differentiated neural stem/progenitor cells were dissociated by enzymatic digestion, permeabilized with Triton X-100, treated with antibodies and then evaluated by a flow cytometer (Beckman-Coulter, Miami, FL, USA)[12-13].

Primary antibodies for immunofluorescence and flow cytometry were as follows: rabbit anti-fibroblast growth factor-8 polyclonal antibody (Bioss), rabbit anti-fibroblast growth factor receptor-3 polyclonal antibody (Bioss), rabbit anti-nestin polyclonal antibody (Boster, Wuhan, China), mouse anti-nestin monoclonal antibody (BD Biosciences Pharmingen™, San Diego, CA, USA), mouse anti-rat neuron specific enolase monoclonal antibody (Millipore, Billerica, MA, USA), rabbit anti-tyrosine hydroxylase antibody (Cell Signaling, Danvers, MA, USA), and mouse anti-rat glial fibrillary acidic protein monoclonal antibody (Sigma, St. Louis, MO, USA).

Antibody neutralization of fibroblast growth factor-8 during neural stem/progenitor cell differentiation in vitro

A rabbit anti-fibroblast growth factor-8 polyclonal antibody was added to differentiation medium every 4 days for 20 days at a final dilution of 1:5 000, and equal amount of H_2O was added to the control. Half-medium volumes were replaced with fresh medium every 8 days. Cells were stained for nestin and observed under a DMIRE2 microscope (Leica) and evaluated by flow cytometry (Beckman-Coulter)[12-13]. The percentages of nestin$^+$ cells (neural stem/progenitor cells) at various differentiation stages were observed to evaluate the rate of differentiation between groups.

Promotion of neural stem/progenitor cell differentiation into neurons and dopaminergic neurons by increasing the fibroblast growth factor-8 concentration in the differentiation medium

We explored the effects of inducing neural stem/progenitor cell differentiation by adding recombinant fibroblast growth factor-8 (50 ng/mL; R&D Systems, Minneapolis, MN, USA) into the differentiation medium Half-medium volumes were replaced with fresh medium every 7 days. At 14 days, ascorbic acid (200 ng/mL; Sigma) was also added to the medium. Immunostaining and flow cytometry were used to analyze the percentages of tyrosine hydroxylase (TH$^+$) cells[12-13]. We also used Annexin V-FITC & PI apoptosis assay kits (Bipec, Cambridge, MA, USA) and flow cytometry to analyze apoptosis at the end of neural differentiation.

Cells were immunostained for nestin, neuron-specific enolase, tyrosine hydroxylase and glial fibrillary acidic

protein, and then observed under a confocal microscope and evaluated by flow cytometry (Beckman-Coulter)[12-13].

Statistical analyses

SPSS 13.0 software (SPSS, Chicago, IL, USA) was used to analyze the data by analysis of variance. Differences between groups were evaluated with the Duncan multiple comparison test. Measurement data were presented as mean ± SD. A value of $P < 0.05$ was considered statistically significant.

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Author contributions: Jiang Lu was responsible for study concept and manuscript authorization, designed, performed and completed this work, analyzed the data and wrote the manuscript. All authors obtained the funding.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animal Ethics Committee, Guangxi University, Hubei University of Medicine in China.

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