EGF/bFGF Promotes Survival, Migration and Differentiation of GFP-Labeled Rhesus Monkey Neural Stem Cells Xenografted into the Rat Brain

Hao Li
Kunming Institute of Zoology Chinese Academy of Sciences
Jingkuan Wei
Kunming University of Science and Technology
Lei Pan
Kunming Municipal Hospital of Traditional Chinese Medicine
Yuhua Zhang
Kunming Institute of Zoology Chinese Academy of Sciences
Liyun Guo
Kunming Medical College: Kunming Medical University
Dongdong Qin
Kunming Municipal Hospital of Traditional Chinese Medicine
Yong Yin
Kunming Medical College: Kunming Medical University
Xintian Hu
Kunming Institute of Zoology Chinese Academy of Sciences
Zhengbo Wang (wangzb@lpbr.cn)
Kunming University of Science and Technology https://orcid.org/0000-0002-4717-5137

Research

Keywords: Neural stem cells, EGF, bFGF, Xenotransplantation, Rat

Posted Date: May 10th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-470057/v1

Abstract

Background: Stem cell replacement therapy is considered a promising treatment for diseases of the central nervous system. Improving the ratio of surviving transplanted cells and increasing the ratio of cells that differentiate into functional neuronal cells are the most important issues related to research on neuroregenerative medicine. Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) have been reported to promote the proliferation and differentiation of neural stem cells (NSCs) *in vitro*, but whether they have the same effect *in vivo* is unclear.

Methods: In this study, NSCs derived from rhesus monkey embryonic stem cells (ESCs) were resuspended in medium with or without EGF/bFGF and xenotransplanted into the rat striatum.

Results: No behavioral abnormalities or teratoma formation were observed in the recipient engrafted rats. GFP-labeled cells exhibited a higher survival rate and longer migration in the EGF/bFGF group than in the control group at 2 months after transplantation. Moreover, the percentages of $Tuj1^+$ neurons and $Map2^+$ neurons in the EGF/bFGF group were significantly higher than those in the control group, while the percentages of astrocytes and oligodendrocytes were significantly lower in the EGF/bGFG group than in the control group.

Conclusions: These findings indicate that EGF/bFGF can promote protrusion of nerve fibers and the survival and neuronal differentiation of transplanted NSCs in the recipient brain, suggesting that EGF/bFGF has a potential application for stem cell therapy.

Introduction

Neural stem cell (NSC) therapy holds great promise for the treatment of neurological diseases (such as traumatic brain injury, neurodegenerative diseases and spinal cord injury). Our previous studies showed that engrafted NSCs can survive, differentiate into neurons[1] and functionally integrate into the host neural circuit[2]. However, the clinical applications of NSC therapy are limited by some critical issues, such as poor survival rates, low differentiation rates, and uncontrollable and unexpected differentiation of engrafted cells in the host brain[3-6]. Interestingly, extracellular signals that originate from the local microenvironment and the presence of growth factors are beneficial for grafted NSCs at the implantation site[7].

Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) are well known to be mitogenic and angiogenic agents[8, 9]. NSCs from embryonic stem cells (ESCs) or the subventricular zone (SVZ) express EGF and bFGF receptors. Therefore, EGF and bFGF are needed to maintain the differentiation potential of these cells in long-term culture[10-12]. These two factors have also been reported to have effects on the expansion, migration and differentiation of endogenous neural progenitors in the both normal and injured brain[13-15]. These findings suggest that EGF and bFGF can serve as benign extracellular factors that promote the survival and differentiation of grafted NSCs and increase the neural regeneration capacity[11, 12].

However, the cellular responses to EGF and bFGF are different both *in vitro* and *in vivo*. For example, EGF and bFGF have differential and site-specific effects on progenitor cells *in vivo*[16] and induce distinct activation time courses of Ras and the mitogen activated protein kinase (MAPK) cascade[17]. This evidence suggests that both EGF and bFGF might have differential effects on the fate of engrafted NSCs in the host brain and that the two factors are necessary for the essential role of engrafted NSCs in NSC therapy. These findings provide a strong impetus for investigating the influence of combined treatment with EGF/bFGF on the survival and differentiation of NSCs after grafting.

In the past, chronic exposure to growth factors was found to raise the risk of uncontrolled tumorigenesis, which is a significant side effect of clinical therapies. Given these findings, the present study was carried out to investigate the influence of short-term exposure to EGF/bFGF on the survival, migration, and differentiation of transplanted NSCs and the protrusion of nerve fibers in the recipient brain.

Materials And Methods

All experimental protocols, including animal care and cell transplantation procedures, were performed according to the approved guidelines (IACUC No. SWYX-2015017) established by the Institutional Animal Care And Use Committee of the Kunming Institute of Zoology. Sprague-Dawley rats were housed under a 12:12-h light/dark cycle with ad libitum access to food and water. GFP-labeled rhesus monkey embryonic stem (LYON-ES) cells were a gift from the Lyon Stem Cell Research Institute (Lyon, France)[18]. All media and chemicals were obtained from Thermo Fisher Scientific Inc. (Beijing, China) unless otherwise specified.

LYON-ES cell culture

LYON-ES cells were cultured in knockout Dulbecco's minimum essential medium (DMEM) supplemented with 20% knockout serum, 1% nonessential amino acids (NEAA), 0.1 mM β-mercaptoethanol (b-ME, Sigma-Aldrich China Inc., Shanghai, China), 2 mM L-glutamine and 5 ng/ml bFGF. CF-1 cells (CF-1-MEF; ATCC, Manassas, USA) were cultured in DMEM supplemented with 2 mM L-glutamine and 15% FBS. LYON-ES cells were cultured with a feeder layer of mitomycin-C-treated mouse embryonic fibroblasts. Mechanical passaging of undifferentiated colonies was performed every 5-7 days by cutting the colonies into large clumps using a flame-pulled Pasteur pipette[2].

NSC differentiation and culture

LYON-ES cell colonies were digested with 1 mg/ml dispase (Sigma-Aldrich) and then washed to remove the dispase. The digested colonies were suspended in N/M medium (50% DMEM/F12; 50% neurobasal medium; 1× N2 supplement; 1× B27 and 2 mM L-glutamine (Sigma-Aldrich)), plated in 15×30 mm dishes coated with agar (Sigma-Aldrich) and allowed to aggregate for 4 days to form embryonic bodies (EBs). After aggregation, EBs were selected and cultured in NP media (DMEM/F12, 1× ITS-x; 2 ng/ml heparin (Sigma-Aldrich)) in a 4-well plate coated with extracellular matrix (Sigma-Aldrich) for 3 to 4 days until

rosettes (NSCs) appeared. The rosettes were mechanically passaged and cultured in NP media containing 20 ng bFGF.

Preparation of cells for transplantation

On the day of transplantation, NSCs ($Nestin^+/Sox1^+/Pax6^+/Map2^-/Tuj1^-$, Fig. 1) were trypsinized into single-cell suspensions. Following three washes in 0.01 M PBS, the cells were resuspended to a concentration of approximately 100,000 cells/μl in 0.01 M PBS or 0.01 M PBS containing EGF (Sigma-Aldrich)/bFGF. All cell suspensions were kept on ice before transplantation, and cell viability and total cell number were estimated before and after the transplantation procedure.

Animals and transplantation

Eighteen six-month-old male Sprague-Dawley rats were divided randomly into two groups. The rats were anesthetized with sodium pentobarbital (0.3% in saline, 35-45 mg/kg, intraperitoneal (i.p.)) and placed in a stereotactic device (Stoelting, United States). An incision was made along the midline to expose the skull, and a 2-mm hole was drilled. A total of 5 μl of a suspension containing 1×10^5 cells per μl of 0.01 M PBS or 0.01 M PBS with 1 ng/μl EGF/bFGF was injected into the rat striatum over a 5-min period with a 5-μl Hamilton syringe. After injection, the needle was held in place for 2 minutes. The following stereotaxic coordinates were used to target the rat striatum: -2 mm anteroposterior from bregma, 4 mm mediolateral, and 6 mm dorsoventral (the schematic location is shown in Fig. 2A). Following NSC transplantation, all animals received daily i.p. injections of 10 mg/kg cyclosporine A (Sigma-Aldrich). Two months after transplantation, the rats were sacrificed by deep anesthesia with pentobarbital (100 mg/kg, i.p.) for brain tissue collection. All animals were injected i.p. with the immunosuppressant cyclosporine A at a dose of 10 mg/kg 2 days prior to transplantation and then injected with the same drug daily at a dose of 10 mg/kg after surgery until sacrifice.

Immunohistochemistry

The rats were transcranially perfused with saline followed by 4% paraformaldehyde (PFA, Aladdin), and then the brain of each rat was removed from the skull and immersed in 4% PFA for four hours. Tissues were cryoprotected in increasing concentrations of sucrose (10%, 20%, and 30%) and then cut into 20-μm-thick slices on a cryostat. The sections were used for fluorescence analysis with an Olympus FV1000 fluorescence microscope (Olympus, Japan). Sections with GFP^+ cells were stained with lineage-specific phenotype markers (Map2, β-tubulin-Ⅲ, O4, and GFAP) as previously reported[2] (table 1, table 2).

Quantification and statistical analysis of the survival and differentiation of grafted cells

Cells on every five 20-μm-thick sections near the transplant site for a total of 20 slides per animal was counted by using ImageJ software. All statistical analyses were performed in a double-blinded manner and carried out by using GraphPad Prism 5 software. Student's t test was used to analyze the results of immunohistochemistry to determine the percentage of NSCs that differentiated into neurons in the two

groups (the $EGF^+/bFGF^+$ group and the $EGF^-/bFGF^-$ group). The data are presented as the means ± SEMs, and the level of significance was set at $P < 0.05$.

Results

Neural progenitor cell identification

LYON-ES cells differentiated into neural rosettes, expanded and could be subcultured while retaining their characteristic morphological and immunocytochemical properties. These cells expressed high levels of the neuroepithelial markers Nestin (Fig. 1A), Sox1 (Fig. 1B) and Pax6 (Fig. 1C) but did not express the neuronal markers Map2 (Fig. 1D) and Tuj1 (Fig. 1E). Neural progenitor cells subsequently differentiated into neurons and glial cells. The viability of $Nestin^+$ cells used for transplantation was over 95%.

Survival and migration of the grafted NSCs

The behavior of grafted neural progenitor cells *in vivo* was assessed two months after transplantation. No behavioral abnormities or teratomas were observed in either the $EGF^+/bFGF^+$ or $EGF^-/bFGF^-$ group.

GFP^+ cells in the brain sections were analyzed using a confocal laser scanning microscope to evaluate the differences in the survival of the grafted NSCs between the rats in the $EGF^+/bFGF^+$ group and those in the vehicle control group. This analysis revealed that there were more GFP^+ cells in the $EGF^+/bFGF^+$ group (Fig. 2B) than in the vehicle control group (Fig. 2C). Moreover, the GFP^+ cells in the $EGF^+/bFGF^+$ group (Fig. 3A, C) formed more nerve fibers than those in the vehicle control group (Fig. 3B, D).

Although the majority of GFP^+ cells in the two groups remained *in situ*, some engrafted cells migrated away from the sites as individual cells or clusters. The distance that the grafted cells migrated away from the implantation sites at two months after the graft in the $EGF^+/bFGF^+$ group was farther than that in the vehicle control. In the $EGF^+/bFGF^+$ group, some grafted cells migrated into the hippocampus (Fig. 2D) and substantia nigra (Fig. 2F), while in the control group, no GFP^+ cells were found in the hippocampus (Fig. 2E), and fewer cells migrated into the substantia nigra than in the $EGF^+/bFGF^+$ group (Fig. 2G).

By analyzing nerve fibers, we found that many nerve fibers from the grafted cells (Fig. 3A) protruded into the host brain. The number of fibers in the experimental group was significantly higher than that in the control group (Fig. 3A, B). In addition, bundles of nerve fibers were observed to extend in the same direction in the $EGF^+/bFGF^+$ group but not in the control group.

Differentiation of grafted NSCs

Transplanted NSCs can differentiate into cells of all three neural lineages (astrocytes, oligodendrocytes and neurons), which can be identified by colabeling transplanted cells with GFP and other neuronal cell type-specific markers. For both groups, cells were labeled with neuronal markers (Tuj1 and Map2), a glia

marker (GFAP) and a oligodendrocyte marker (O4), and it was found that the grafted neural progenitors differentiated into neurons (Fig. 4A, B, C, D), glial cells (Fig. 4E, F) and oligodendrocytes (Fig. 4G, H).

Detailed quantitative analysis of the phenotypes of NSCs grafted into rat brains was performed by colabeling the transplanted cells with GFP and other nerve cell type-specific markers. The results revealed that the percentage of cells that differentiated into glial cells was higher than the percentage of cells that differentiated into neurons in both engrafted groups. A small proportion of the grafted cells were $Map2^+$ (11.87% and 16.37% in the vehicle control and $EGF^+/bFGF^+$ groups, respectively; Fig. 5). A total of 19.67% and 33.47% grafted cells were $Tuj1^+$- in the vehicle control and $EGF^+/bFGF^+$ groups, respectively (Fig. 5). In the vehicle control and $EGF^+/bFGF^+$ groups, 44.13% and 35.23% of grafted cells, respectively, were $GFAP^+$ (Fig. 5), and some of the grafted cells were $O4^+$ (35.23% and 20.1% for the vehicle control and $EGF^+/bFGF^+$ groups, respectively; Fig. 5). Nerve cell type-specific differentiation of the engrafted cells was significantly different between the two groups.

Discussion

Transplanted cells exhibit poor survival rates, low differentiation rates, and uncontrollable and unexpected differentiation, which limits the clinical applications of cell transplantation. Growth factors can regulate cell proliferation and determine cell fate. However, the effects of growth factors on exogenously transplanted NSCs in the recipient brain are complicated and depend on the specific signaling pathways activated in the cells. In this study, the effects of short-term combined treatment with EGF/bFGF on the fate of transplanted NSCs in the recipient brain were evaluated by adding EGF/bFGF to suspensions of NSCs before transplantation. Compared with vehicle control, EGF/bFGF increased the survival rate, migration distance, and neuronal differentiation of transplanted NSCs and enhanced the protrusion of nerve fibers from these cells in the rat brain.

Postmortem histological evaluation of the brains of recipient rats revealed that more GFP^+ cells were observed in the $EGF^+/bFGF^+$ group than in the vehicle control group. This suggested that EGF/bFGF promoted the survival of the grafted NSCs. In addition, the morphology of the GFP^+ cells was more neuron-like in the $EGF^+/bFGF^+$ group than in the vehicle control group (Fig. 3C, D). These findings are consistent with previous studies, showing that bFGF, EGF and nerve growth factor (NGF) can promote the survival of cultured neurons *in vitro*[19] and the proliferation of newborn neurons in the adult rat brain *in vivo*[20] and transplanted progenitors in recipients[20-25]. The mechanisms underlying these effects are unclear. It is believed that the primary function of growth factors is the regulation of metabolic glucose uptake and thus the maintenance of mitochondrial homeostasis and activation of anabolic pathways required for cell growth[26].

The increased survival rate of the grafted NSCs was attributed to the combined treatment effects of EGF/bFGF in the recipient brain, which can overcome the poor survival rate of transplanted cells and promote the effect of stem cell replacement therapy[4, 6, 27, 28].

In this study, EGF/bFGF was found to promote the migration of grafted NSCs. Some grafted cells migrated into the hippocampus and substantia nigra in the $EGF^+/bFGF^+$ group, while only very few cells were found in these regions in the control group. These findings are consistent with *in vivo* studies showing that EGF and bFGF induce the migration of neural progenitors in the SVZ of adult animals towards the olfactory bulb or throughout the injured brain[16, 29]. The specific mechanisms underlying this effect need to be further elucidated.

Immunohistochemical analysis showed higher expression of neuronal markers and lower expression of glial markers in the $EGF^+/bFGF^+$ treatment group than in the vehicle control group, suggesting that EGF/bFGF promotes neuronal differentiation of grafted NSCs. These findings are consistent with earlier studies showing that growth factors (e.g., EGF and bFGF) promote the neuronal differentiation of cultured stem cells, endogenous progenitors and transplanted neural progenitors [30-32]. The mechanisms underlying this neuronal differentiation might be associated with the MAPK/Erk signaling pathway because MAPK has been shown to be activated indirectly by extracellular growth factors[33]. It is important to note that an increase in the neuronal differentiation rate of engrafted NSCs is beneficial for stem cell therapy because neurons are more beneficial to the injured brain than glial cells. However, if transplantation is performed to improve the environment for host neurons, it may be preferable for the transplanted cells to differentiate into glial cells (due to neuroprotective and anti-inflammatory effects).

The cell survival and neuronal differentiation of transplanted NSCs in the $EGF^+/bFGF^+$ group were significantly higher than those of transplanted NSCs in the vehicle control group. However, the specific mechanisms involved in these effects have not been identified. Furthermore, it would be informative to investigate the effects of growth factors on transplanted cells in models of brain diseases.

Conclusion

EGF and bFGF can promote the survival, migration and neuronal differentiation of grafted NSCs in the host brain and may have potential therapeutic applications in stem cell therapy.

Abbreviations

bFGF basic fibroblast growth factor

EGF epidermal growth factor

NSCs neural stem cells

ESCs embryonic stem cells

SVZ subventricular zone

MAPK mitogen activated protein kinase

EBs embryonic bodies

GFAP glial fibrillary acidic protein

GFP green fluorescent protein

NGF nerve growth factor

Declarations

Availability of data and materials

The data used to support the findings of this study are included within the article.

Acknowledgements

Funding

This work was supported by the National Program on Key Basic Research Projects (2018YFA0801403), the National Natural Science Foundation of China (31960120), the Applied Basic Research Programs of Science and Technology Commission Foundation of Yunnan Province (2018FB052), and the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB32060200).

Author information

Hao Li, Jingkuan Wei and Lei Pan contributed equally to this work.

Contributions

ZW, XH and YY conceived and designed the experiments. ZW performed the experiment. ZW, HL and JW analyzed and discussed the data. ZW, HL and LP wrote the paper. YZ and LP write-up help/proof reading, LG, DQ and YY gave proof reading; ZW, XH and JW were project managers and gave financial support. All authors contributed to the editing of the paper and to scientific discussions.

Ethics declarations

Ethics approval and consent to participate

All experimental protocols, including animal care and cell transplantation procedures, were performed according to the approved guidelines (IACUC No. SWYX-2015017) established by the Institutional Animal Care And Use Committee of the Kunming Institute of Zoology.

Consent for publication

Not applicable.

Competing interests

The authors have no competing financial interests and non-financial competing interests to declare.

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Tables

Table 1 Primary antibodies used for immunocytochemistry and immunohistochemistry

Immunogen	Species/Class	Cat. No	Supplier	Dilution For ICC
GFAP	Mouse/IgG1	SMI-21R	Convance	1:1000
Map2	Mouse/IgG1	M4403	Sigma	1:800
Nestin	Rabbit/IgG	AB5922	Millipore	1:200
NeuN	Mouse/IgG1	MAB377	Millipore	1:500
NF-200	Mouse/IgG1	MAB5266	Millipore	1:400
O4	Mouse/IgM	MAB1326	R&D	1:500
PAX6	Mouse/IgG1	Pax6	Chemicon	1:200
Sox1	Rabbit/IgG	Ab87775	Abcam	1:800
Tau-5	Mouse/IgG1	Ab80579	Abcam	1:1000
Tuj1	Mouse/IgG1	MAB1637	Millipore	1:200

Table 2 Secondary antibodies used for immunocytochemistry and immunohistochemistry

Name	Supplier	Dilution
Goat anti-mouse IgG-Cy3	Sigma	1:400
Goat anti-rabbit IgG-Cy3	Sigma	1:400

Figures

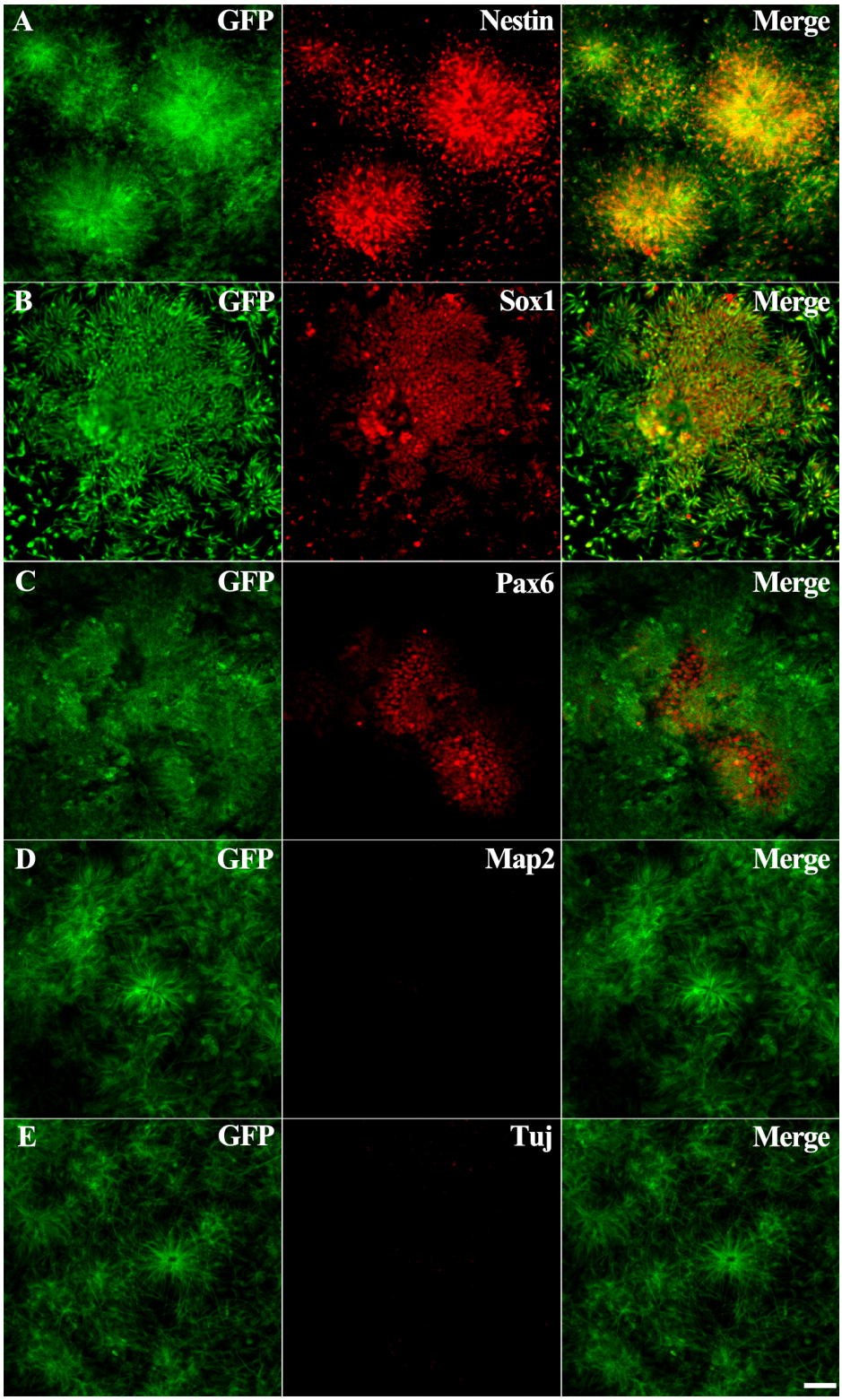

Figure 1

Immunocytochemical tests of the purity of NSCs. (A) Nestin was used to identify NSCs, and the image suggests that the majority of ESCs differentiated into NSCs. (B) Sox1 was used to identify NSCs, and the results suggested that most of the rosette cells were NSCs. (C) Pax1 was used to identify NSCs, and the results suggested that most of the cells were NSCs. (D) Map2 was used to identify neurons, and the results showed that no NSCs differentiated into neurons. (E) Beta tubulin III was used to identify neurons, and the image suggests that no NSCs differentiated into neurons. Scale bar: 50 μm.

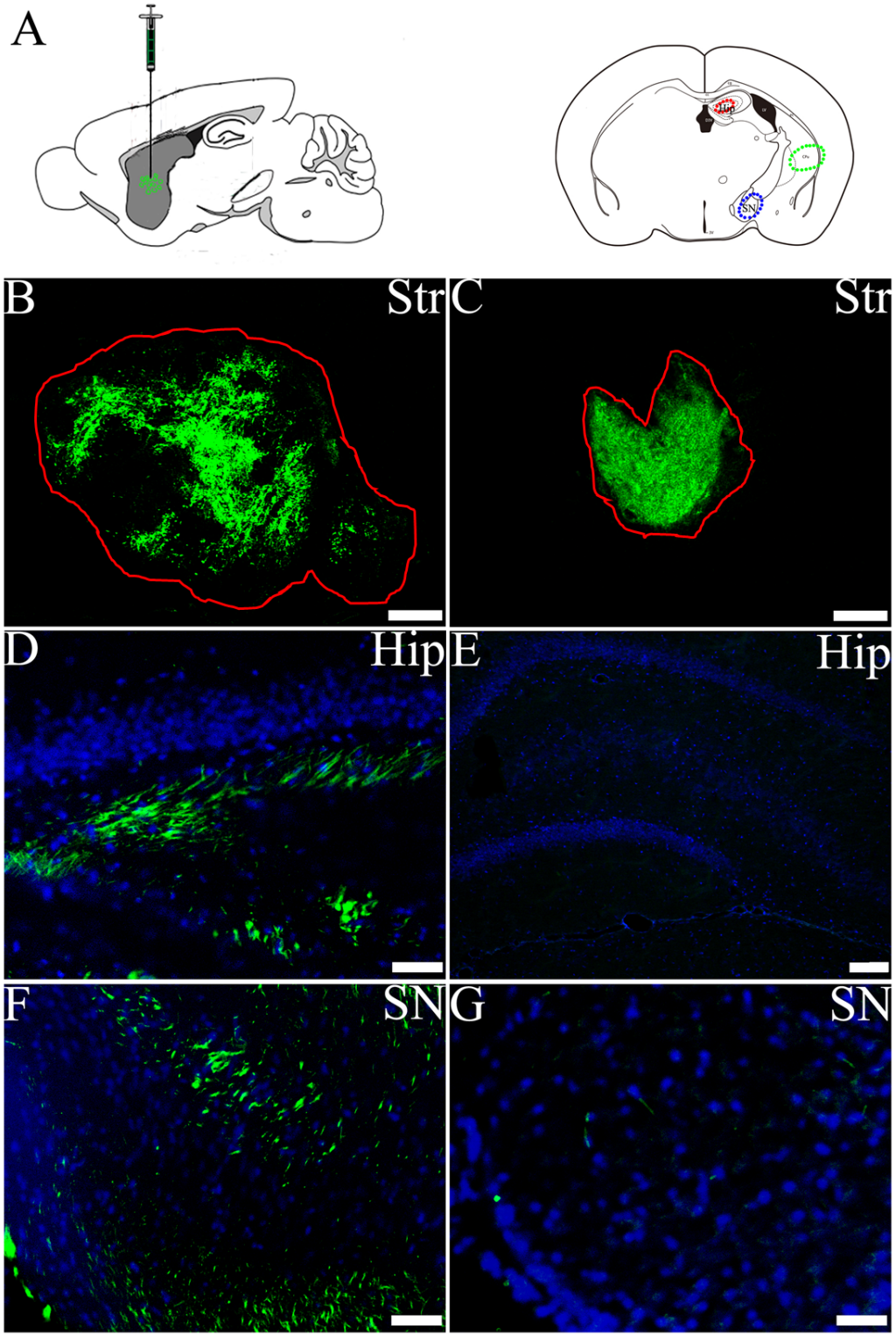

Figure 2

EGF/bFGF promotes the survival and migration of transplanted NSCs in the rat brain after 2 months. (A) Patterns of transplanted stem cells in the striatum. A sagittal section (left) showing the cell transplantation site; a coronal section (right, from the brain of a rat from the EHF/bFGF group that underwent cell transplantation) showing the cell transplantation site (striatum (Str)) and the migration sites (hippocampus (Hip) and substantia nigra (SN)). (B) Representative confocal images of transplanted NSCs (labeled with GFP, green, region outlined in red) near the transplantation site in the EGF/bFGF group. (C) Representative confocal images of transplanted NSCs (labeled with GFP, green) near the transplantation site in the vehicle control group. (D) Some of the transplanted NSCs (labeled with GFP, green) migrated into the hippocampus in the EGF/bFGF group. (E) Almost no transplanted NSCs (labeled with GFP, green) migrated into the hippocampus in the vehicle control group. (F) Some transplanted NSCs (labeled with GFP, green) migrated into the substantia nigra in the EGF/bFGF group. (G) Few transplanted NSCs (labeled with GFP, green) migrated into the substantia nigra in the vehicle control group. Scale bar: A 2000 μm; B, C: 250 μm; D, F, G: 50 μm; E: 100 μm

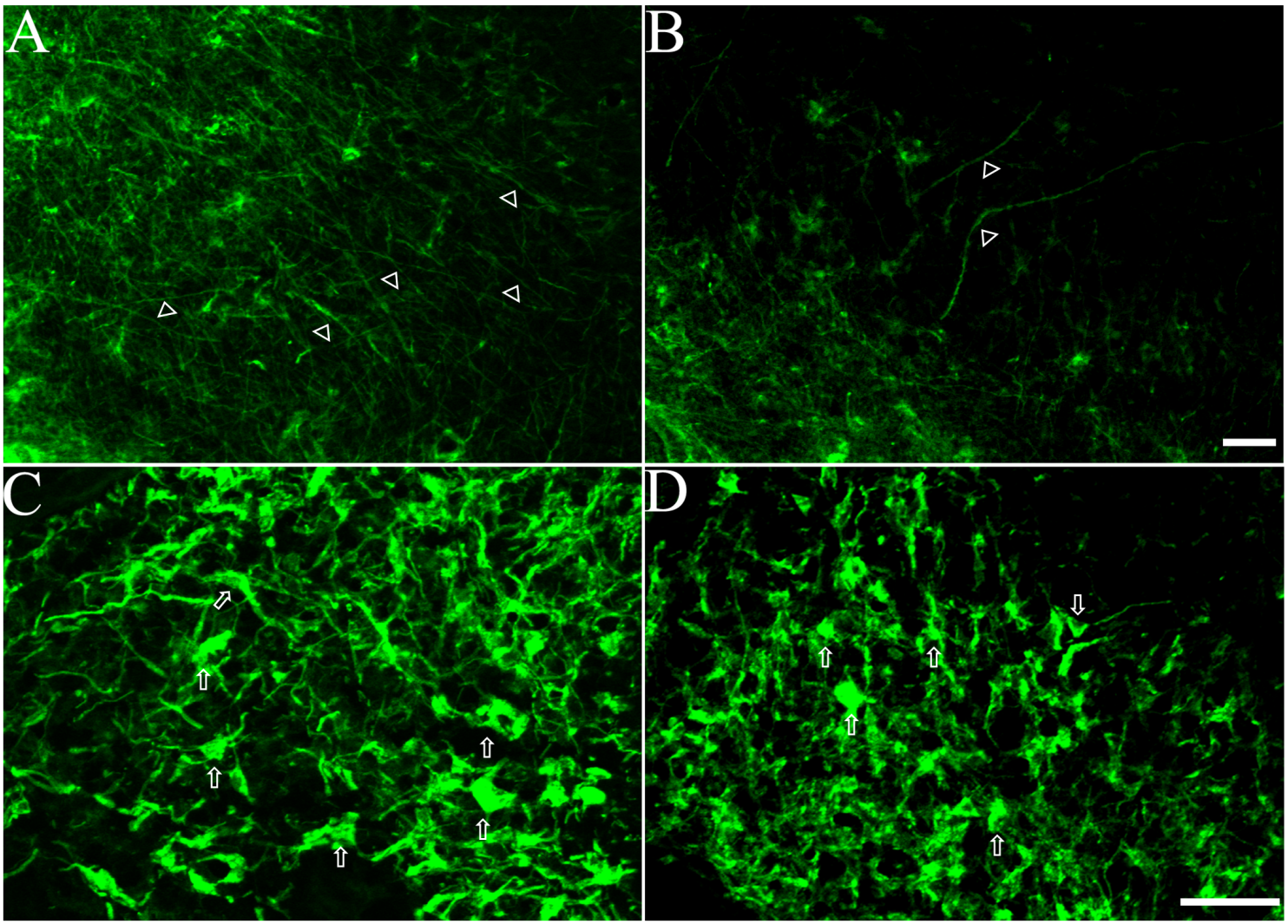

Figure 3

Morphology of the surviving transplanted NSCs in the rat brain. (A) Representative confocal image showing that many transplanted NSCs (labeled with GFP, green) projected into the surrounding host tissue (arrowhead) in the EGF+/bFGF+ group. (B) Representative confocal image showing that fewer transplanted NSCs (labeled with GFP, green) projected into the surrounding host tissue (arrowhead) in the vehicle control group than in the EGF+/bFGF+ group. Representative confocal images showing that surviving cells (arrow) extended more processes in the EGF+/bFGF+ group (C) than in the vehicle control group (D). Scale bar: 50 μm.

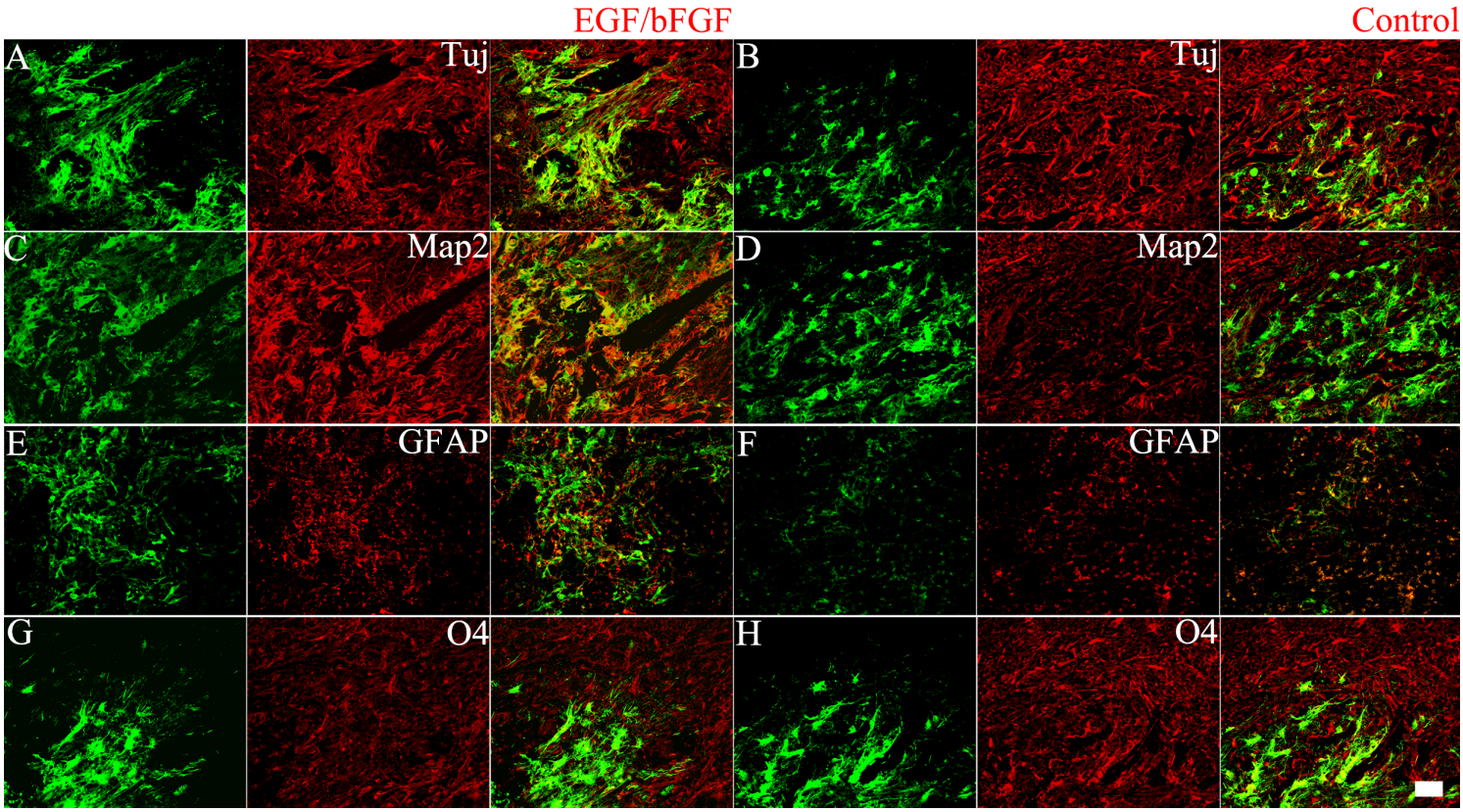

Figure 4

Identification of neuronally differentiated engrafted cells 2 months after transplantation. Engrafted cells differentiated into neurons, astrocytes and oligodendrocytes in the EGF+/bFGF+ (A, C, E, G) and vehicle control groups (B, D, F, H). (A) and (B) show that the transplanted NSCs differentiated into neurons (Tuj). (C) and (D) show that the transplanted NSCs differentiated into neurons (Map2). (E) and (F) show that the transplanted NSCs differentiated into astrocytes (GFAP). (G) and (H) show that the transplanted NSCs differentiated into oligodendrocytes (O4). Scale bar: 30 μm.

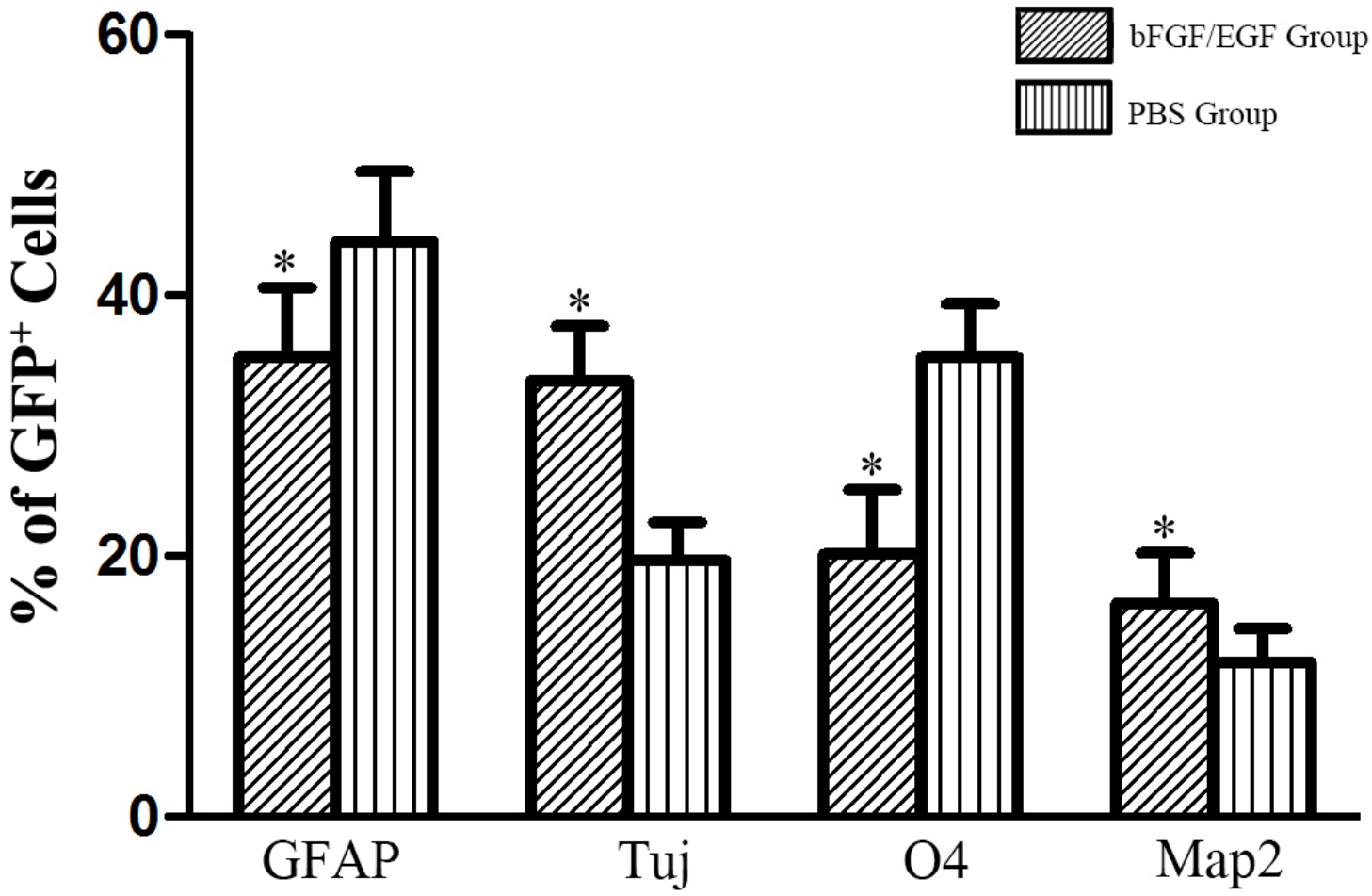

Figure 5

Double immunohistochemistry for quantitative analysis of different cell types 2 months after transplantation. Short-term treatment with EGF/bFGF significantly increased the percentage of transplanted NSCs that differentiated into neurons in the host brain. Map2, Tuj1, GFAP, and O4 represent mature neurons, neurons, astrocytes and oligodendrocytes, respectively. All values are represented as the mean ± S.E.M. *P < 0.05; **P < 0.01; ***P < 0.001.