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

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

**License:** This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
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/bFGF 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 pipette².

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 abnormalities 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.

References

1. Dong JR, Guo LY, Qu JG, Qi RL, Wang WC, Xiao CJ, Wang ZB: [Rhesus monkey embryonic stem cells differentiation, proliferation and allotransplantation]. Dongwuxue Yanjiu 2012, 33(1):43-48.
2. Wei JK, Wang WC, Zhai RW, Zhang YH, Yang SC, Rizak J, Li L, Xu LQ, Liu L, Pan MK et al: Neurons Differentiated from Transplanted Stem Cells Respond Functionally to Acoustic Stimuli in the Awake Monkey Brain. Cell Rep 2016, 16(4):1016-1025.
3. Bacigaluppi M, Russo GL, Peruzzotti-Jametti L, Rossi S, Sandrone S, Butti E, De Ceglia R, Bergamaschi A, Motta C, Gallizzioli M et al: Neural Stem Cell Transplantation Induces Stroke Recovery by Upregulating Glutamate Transporter GLT-1 in Astrocytes. J Neurosci 2016, 36(41):10529-10544.
4. Hicks AU, Lappalainen RS, Narkilahti S, Suuronen R, Corbett D, Sivenius J, Hovatta O, Jolkkonen J: Transplantation of human embryonic stem cell-derived neural precursor cells and enriched environment after cortical stroke in rats: cell survival and functional recovery. Eur J Neurosci 2009, 29(3):562-574.
5. Kikuchi T, Morizane A, Doi D, Magotani H, Onoe H, Hayashi T, Mizuma H, Takara S, Takahashi R, Inoue H et al: Human iPS cell-derived dopaminergic neurons function in a primate Parkinson’s disease model. Nature 2017, 548(7669):592-596.
6. Sortwell CE, Pitzer MR, Collier TJ: Time course of apoptotic cell death within mesencephalic cell suspension grafts: implications for improving grafted dopamine neuron survival. Exp Neurol 2000, 165(2):268-277.
7. Liang Y, Agren L, Lyczek A, Walczak P, Bulte JW: Neural progenitor cell survival in mouse brain can be improved by co-transplantation of helper cells expressing bFGF under doxycycline control. Exp Neurol 2013, 247:73-79.
8. Mason I: Initiation to end point: the multiple roles of fibroblast growth factors in neural development. Nat Rev Neurosci 2007, 8(8):583-596.
9. Parson-Wingerter P, Elliott KE, Clark JI, Farr AG: Fibroblast growth factor-2 selectively stimulates angiogenesis of small vessels in arterial tree. Arterioscler Thromb Vasc Biol 2000, 20(5):1250-1256.
10. Huma T, Hu X, Ma Y, Willden A, Rizak J, Shahab M, Wang Z: Kisspeptin-10 treatment generated specific GnRH expression in cells differentiated from rhesus monkey derived Lyon NSCs. Neuroscience 2017, 349:318-329.
11. Kelly CM, Tyers P, Borg MT, Svendsen CN, Dunnett SB, Rosser AE: EGF and FGF-2 responsiveness of rat and mouse neural precursors derived from the embryonic CNS. Brain Res Bull 2005, 68(1-2):83-94.
12. Supeno NE, Pati S, Hadi RA, Ghani AR, Mustafa Z, Abdullah JM, Idris FM, Han X, Jaafar H: IGF-1 acts as controlling switch for long-term proliferation and maintenance of EGF/FGF-responsive striatal
neural stem cells. Int J Med Sci 2013, 10(5):522-531.

13. Jin K, LaFevre-Bernt M, Sun Y, Chen S, Gafni J, Crippen D, Logvinova A, Ross CA, Greenberg DA, Ellerby LM: FGF-2 promotes neurogenesis and neuroprotection and prolongs survival in a transgenic mouse model of Huntington's disease. Proc Natl Acad Sci U S A 2005, 102(50):18189-18194.

14. Nakatomi H, Kuriu T, Okabe S, Yamamoto S, Hatano O, Kawahara N, Tamura A, Kirino T, Nakafuku M: Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. Cell 2002, 110(4):429-441.

15. Tureyen K, Vemuganti R, Bowen KK, Sailor KA, Dempsey RJ: EGF and FGF-2 infusion increases post-ischemic neural progenitor cell proliferation in the adult rat brain. Neurosurgery 2005, 57(6):1254-1263; discussion 1254-1263.

16. Kuhn HG, Winkler J, Kempermann G, Thal LJ, Gage FH: Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. J Neurosci 1997, 17(15):5820-5829.

17. Yamada S, Taketomi T, Yoshimura A: Model analysis of difference between EGF pathway and FGF pathway. Biochem Biophys Res Commun 2004, 314(4):1113-1120.

18. Wianny F, Bernat A, Huissoud C, Marcy G, Markossian S, Cortay V, Giroud P, Leivel V, Kennedy H, Savatier P et al: Derivation and cloning of a novel rhesus embryonic stem cell line stably expressing tau-green fluorescent protein. Stem Cells 2008, 26(6):1444-1453.

19. Walicke P, Cowan WM, Ueno N, Baird A, Guillemin R: Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. Proc Natl Acad Sci U S A 1986, 83(9):3012-3016.

20. Frielingsdorf H, Simpson DR, Thal LJ, Pizzo DP: Nerve growth factor promotes survival of new neurons in the adult hippocampus. Neurobiol Dis 2007, 26(1):47-55.

21. Bhang SH, Lee YE, Cho SW, Shim JW, Lee SH, Choi CY, Chang JW, Kim BS: Basic fibroblast growth factor promotes bone marrow stromal cell transplantation-mediated neural regeneration in traumatic brain injury. Biochem Biophys Res Commun 2007, 359(1):40-45.

22. Darsalia V, Kallur T, Kokaia Z: Survival, migration and neuronal differentiation of human fetal striatal and cortical neural stem cells grafted in stroke-damaged rat striatum. Eur J Neurosci 2007, 26(3):605-614.

23. Fricker-Gates RA, Winkler C, Kirik D, Rosenblad C, Carpenter MK, Bjorklund A: EGF infusion stimulates the proliferation and migration of embryonic progenitor cells transplanted in the adult rat striatum. Exp Neurol 2000, 165(2):237-247.

24. Meng XT, Li C, Dong ZY, Liu JM, Li W, Liu Y, Xue H, Chen D: Co-transplantation of bFGF-expressing amniotic epithelial cells and neural stem cells promotes functional recovery in spinal cord-injured rats. Cell Biol Int 2008, 32(12):1546-1558.

25. Xiong N, Yang H, Liu L, Xiong J, Zhang Z, Zhang X, Jia M, Huang J, Zhang Z, Mohamed AA et al: bFGF promotes the differentiation and effectiveness of human bone marrow mesenchymal stem cells in a rotenone model for Parkinson's disease. Environ Toxicol Pharmacol 2013, 36(2):411-422.
26. Vander Heiden MG, Plas DR, Rathmell JC, Fox CJ, Harris MH, Thompson CB: Growth factors can influence cell growth and survival through effects on glucose metabolism. Mol Cell Biol 2001, 21(17):5899-5912.

27. Bacigaluppi M, Pluchino S, Martino G, Kilic E, Hermann DM: Neural stem/precursor cells for the treatment of ischemic stroke. J Neurol Sci 2008, 265(1-2):73-77.

28. Politis M, Lindvall O: Clinical application of stem cell therapy in Parkinson's disease. BMC Med 2012, 10:1.

29. Baldauf K, Reymann KG: Influence of EGF/bFGF treatment on proliferation, early neurogenesis and infarct volume after transient focal ischemia. Brain Res 2005, 1056(2):158-167.

30. Craig CG, Tropepe V, Morshhead CM, Reynolds BA, Weiss S, van der Kooy D: In vivo growth factor expansion of endogenous subependymal neural precursor cell populations in the adult mouse brain. J Neurosci 1996, 16(8):2649-2658.

31. Fricker RA, Carpenter MK, Winkler C, Greco C, Gates MA, Bjorklund A: Site-specific migration and neuronal differentiation of human neural progenitor cells after transplantation in the adult rat brain. J Neurosci 1999, 19(14):5990-6005.

32. Schuldiner M, Yanuka O, Itskovitz-Eldor J, Melton DA, Benvenisty N: Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A 2000, 97(21):11307-11312.

33. Shaul YD, Seger R: The MEK/ERK cascade: from signaling specificity to diverse functions. Biochim Biophys Acta 2007, 1773(8):1213-1226.

Tables

Table 1 Primary antibodies used for immunocytochemistry and immunohistochemistry

| Immunogen | Species/Class | Cat. No | Supplier | Dilution For ICC |
|-----------|---------------|---------|----------|-----------------|
| GFAP      | Mouse/IgG1    | SMIF21R | Covance  | 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**

A  | GFP | Nestin | Merge |
---|-----|--------|-------|
B  | GFP | Sox1   | Merge |
C  | GFP | Pax6   | Merge |
D  | GFP | Map2   | Merge |
E  | GFP | Tuj    | Merge |
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 EGF/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.