Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Zhu J, Zhou L, XingWu F. Tracking neural stem cells in patients with brain trauma.
N Engl J Med 2006;355:2376-8.

Supplementary Information

1. Isolation procedure of NSCs

About 5-20g exposed brain tissues, which were usually contused and mixed with broken hair and blood among the cranial fracture bones, but sometimes just in the hair, were collected directly into cold DMEM-F12-N2 Medium (Gibco) containing 20% FBS, and rinsed five times. Because debris of brain tissue no longer retained its normal architecture, it was difficult to distinguish sub-cortical structure from cortical structure. But in some cases, we obtained large pieces of brain tissue, in which we could distinguish cortex from sub-cortical structure. After resection and removal of pia and arachnoid tissues, specimens were minced into slurries of 100–700 μl with sterile scalpel blades, and incubated in prewarmed papain/DNase I solution (final concentrations: 0.114U/ml papain; 0.1 U/ml DNase; Sigma) for 30 minutes at 37°C. The resulting crude tissue homogenate was plated into uncoated flask with complete medium. We used complete medium for NSC culture: a mixture of 44 ml of Neurobasal (NB) medium (Gibco), 0.5 ml of glutamine, 0.5 ml of B27 (Gibco), 5 ml of fetal calf serum, 0.04 ml of gentamicin (Cellgro) and supplemented with epidermal growth factor (EGF) (20ng/ml; Sigma) and fibroblast growth factor-2 (FGF2) (20ng/ml; Sigma). After plating, 50% of the medium was replaced, 2 times weekly. Non-adherent cells and debris from the removed supernatant were pelleted by centrifugation and re-introduced into the cultures together with the fresh medium[1]. After 7 days in culture, plates were agitated by sharp rapping with a marking pen and 100% of the culture medium and non-adherent material was removed; the removed medium was centrifuged to pellet cell debris and non-adherent cells and to recover complete medium as supernatant. After one week, the procedure was repeated, except that the non-adherent fraction was discarded. In this way, an additional population of cells was recovered from the non-adherent fraction. All the cells were eventually combined to form a single population of cultured cells.

We succeeded in isolating neural progenitor cells (NPCs)/NSCs in the 16 cases out of 22 adult patients from the injured brain tissues and propagate them in vitro. Among 16 cases, NPCs/NSCs were isolated from frontal lobes in 6 cases, from parietal lobes in 7 cases, from temporal lobe in 2 cases, from occipital lobe in 1 case. This suggests that debris of brain tissue in open head injury, at least in some cases, can serve as a source of NPCs/NSCs. We failed to culture NPCs from 6 cases, which we attribute to (i) contamination of brain tissue (ii) exposure outside of the brain for a long time (the average exposure time was 8.1 hours in failed cases and 3.3 hours in successful cases), and (iii) inadequate quantity of brain tissue. We found that it is critical not to let the brain tissues dry, and to keep them in the collecting medium containing 20% FBS under low temperature (4-8°C) for transfer to culture medium.

2. Biochemical and physical characteristics of the NSCs

Clonal assay:
Clonogenic studies were done to confirm that the progenitor cells possessed self-renewal properties. Single spheres were dissociated into single cells after about 30 days of culture and plated in a 96-well plate with 0.5 ml/well of DMEM-F12-N2 with bFGF/PDGF-AA.

Seven days later, the cells were switched to serum free medium with bFGF alone (30 ng/ml). Over the next 7 days, we observed the formation of neurospheres—spherical masses of cells that expand from single parental progenitors, and a cloning efficiency of 3.5–3.9%. The neurospheres were typically >150μm in diameter and included 68±18.2 cells/sphere. For secondary cloning, the neurospheres were passaged at the 50- to 100-cell stage, by dissociation to single cells, 2.5% of the secondly seeded cells gave rise to a new culture of neural stem cells, which could reach 3–5 x 10^5 cells after 40 days. This clonogenic potential was observed in samples derived from10 of the 16 individuals studied. Furthermore, each neurosphere can give rise to a large clonal expansion in the numbers of sphere (average number of secondary spheres from each primary sphere = 81.2±5.6). However, over subsequent passages up to passage 12, the numbers of new spheres that formed from dissociation of each single sphere dropped. The proliferation rate of the cells derived from frontal lobes was higher than that of other region. We found that NSCs isolated from sub-cortex proliferated significantly more strongly than those isolated from cortical matter. ($P<0.01$).

Differentiation and Immunostaining:
To assay the differentiation potential of the adult human NSCs, individual clonally derived spheres were plated as whole spheres or dissociated spheres in 24-well plates on a substrate of poly-L-ornithine plus laminin (BD Biosciences) in DMEM-F12-N2 medium with 15% fetal calf serum. Media were changed every 3–4 days, and cells were allowed to migrate and differentiate over the course of 2 weeks, at which time the plates were fixed in fresh 4% paraformaldehyde. Fixed cells were incubated for 15 min in a blocking solution containing 0.2% Triton X-100, and 5% normal goat serum before incubation with the primary antibody. Immunohistochemical analysis of the cells was tested by using antibodies directed to specific markers. Nestin and Pax6 was used for undifferentiated cells; beta-tubulinIII, microtubule-associated protein 2 (MAP2) and neurofilament-M were used for neuron; glial fibrillary acidic protein (GFAP) was used for glia; GalC and the O4 sulfatide antigen were used for oligodendrocytes. Under these culture conditions, the spheres consisted of 8.9±2.3% βIII-tubulin+ neurons, 61.3±5.7% GFAP+ astrocytes and 5.8±1.7% O4+ oligodendrocytes. Strikingly, the percentage of neurons derived from different regions differed significantly. Cultures derived from temporal and occipital lobes gave rise to 8.2±1.2% and 4.3±0.7% neurons, respectively (n=3), whereas 5.0±1.1% of the cells derived from parietal lobes were βIII-tubulin+ (n=7). A higher proportion of neurons (11.9±1.9%; n=6) was generated from tissue excised from frontal lobes.

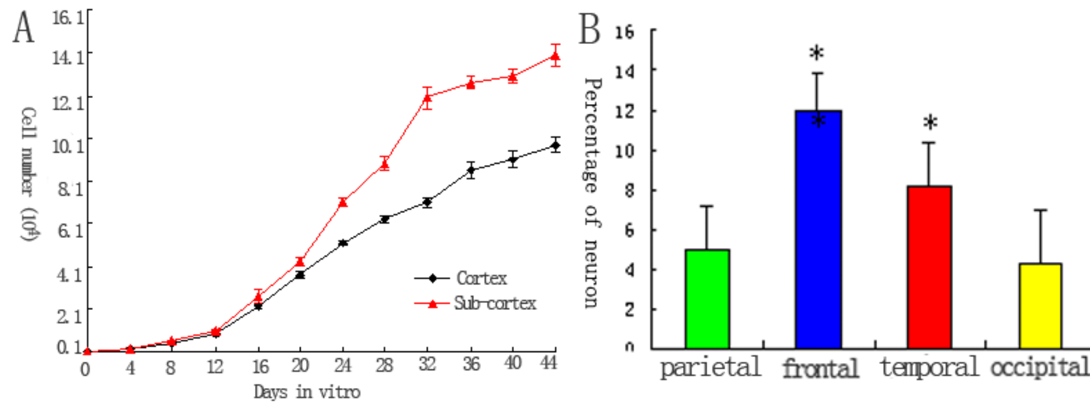

Fig. 1. A. NSCs isolated from sub-cortex proliferated more strongly than those isolated from cortical matter (P<0.01). B. The percentage of neurons derived from different regions differed. Cultures derived from temporal and occipital lobes had 8.2±1.2% and 4.3±0.7% neurons, respectively (n=3), whereas, 5.0±1.1% of the cells derived from parietal lobes were βIII-tubulin+ (n=7). A higher proportion of neurons (11.9±1.9%; n=6) was generated in frontal lobes. asterisk: $P < 0.01$

Electrophysiology Characteristics:

We performed electrophysiological experiments to investigate if the adult NSCs give rise to functional neurons in vitro and in vivo. It will be crucial to determine the functional significance of these newly generated cells in the brain. Whole-cell recordings were obtained with an Axonclamp200B amplifier (Axon Instruments). Cells were visualized on an inverted Nikon microscope with mercury arc lamp attachment. A holding potential of -70 mV and voltage steps of 10 mV with 100 ms durations were applied to the recorded cells through the patch electrodes. Resting membrane potentials was -51.46±8.12mV. Action potentials were recorded (Fig.3A). APa was 39.23±5.06m; APd was10.14±1.25ms. A total of 47 NSCs-derived cells were recorded, in 4 cultures derived from 4 patients. Of these, 21 showed voltage-activated sodium ion currents (INa) of >100 nA, and 9 had INa > 600 (Fig.3B), compatible with the fast sodium currents of neuronal depolarization. Accordingly, whereas two of seven cells with INa > 800 generated stimulus-evoked action potentials, none did so with INa < 800. In addition, none of morphologically non-neuronal cells showed substantial current-induced sodium currents.

For in vivo study, we use a virus vector expressing green fluorescent protein (GFP) to labels NSCs, and that can be visualized in live brain slice. Adult human NSCs labeled with GFP gene were grafted into brain of nude mice. Eight weeks after transplantation, brains of the mice were removed into a chilled solution containing: 110 mM choline Cl^-, 2.5 mM KCl, 1.3 mM NaH_2PO_4, 25 mM $NaHCO_3$, 0.5 mM $CaCl_2$, 7 mM $MgCl_2$, 20 mM dextrose, 1.3 mM Na^+ ascorbate, 0.6 mM Na^+ pyruvate, 5.5 mM kynurenic acid. Brain slices were cut into sections of 200-400 µm, transferred to a chamber containing artificial cerebrospinal fluid (124 mM NaCl, 5 mM KCl, 26 mM $NaHCO_3$, 0.1 mM $CaCl_2$, 1.3 mM $MgCl_2$, 10 mM D-glucose), bubbled with 95% O_2/5% CO_2 (pH 7.4, 320 mOsm), and stored at 30°C. Using fluorescent optics, slices were scanned for GFP+ cells. Microelectrodes (6±8MQ) were pulled from borosilicate glass and filled with 140 mM potassium gluconate, 11 mM EGTA 10 mM HEPES, 35 mM KOH and 1 mM $CaCl_2$ (pH 8.2; 300 mOsm). Criteria to include cells in the analysis were: (1) visual confirmation of GFP in the pipette tip; (2) resting potential less than -50mV; and (3) leak current less than

300 pA. The action potential or continuous K+ currents were recorded by patch clamp in the GFP-positive neurons derived from adult NSCs following transplantation in vivo (Fig.3C).

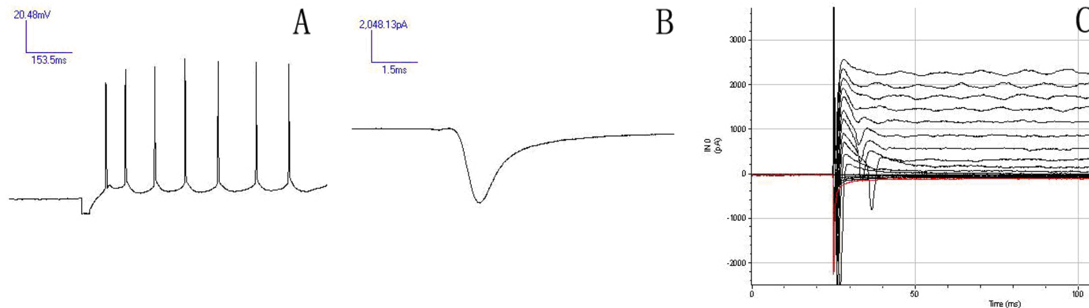

Fig. 2 (A) Action potentials of the neurons derived from NSCs was recorded by whole cell patch clamp. (B) Inward Na+ current in neurons derived from NSCs was recorded in vitro. (C) Continuous K+ currents recorded by patch clamp in a GFP positive neuron derived from NSC in vivo.

3. Gene transfer experiment to distinguish MRI signal generated by neural stem cell migration from MRI signal potentially generated by macrophages

It is important to differentiate between true migration of SPIO-labeled NSC to the site of injury and the possibility that the host's macrophages engulfed the foreign cells, acquired the tracking label, and then tracked to the injury site.

A retroviral marker gene is expressed only in live cells, and not dead cells or macrophages that may engulf the foreign cells that express the marker gene. We thus used a retroviral construct containing the gene that encodes the green fluorescent protein (GFP) to distinguish between SPIO-labelled NSCs, and the possibility that magnetic signal is generated by macrophages that have engulfed the labeled NSCs.
We transduced NSCs with retroviral green fluorescent protein (GFP), and selected the GFP-positive NSCs for SPIO labeling. Then the GFP and SPIO double-labeled NSCs were contra-laterally implanted into a rat brain modelling traumatic brain injury. An identical staining pattern of GFP and Prussian blue (the latter indicating SPIO) indicates that the hypointensity signals in MRI are derived from the SPIO-labeled NSCs, rather than macrophages. The experiment was conducted as follows. First, we established traumatic brain injury in rats. One week later, SPIO and GFP double-labeled NSCs were contra-laterally implanted into the brain. Weekly MR imaging showed that the hypointensity signal migrated from the implantation site to the injured region during the 3 weeks of follow-up (Fig. 3). Histology confirmed that a large number of Prussian blue positive cells had migrated toward the lesion at 21 days after implantation, corresponding to the planes of the MRI investigation (Fig. 4). In parallel, the GFP labeled cells in coronal brain sections were consistent with Prussian blue positive cells (Fig. 5). We therefore conclude that the hypointensity signals did not come from engulfment of the implanted cells by macrophages, but rather, from SPIO-labeled NSCs.

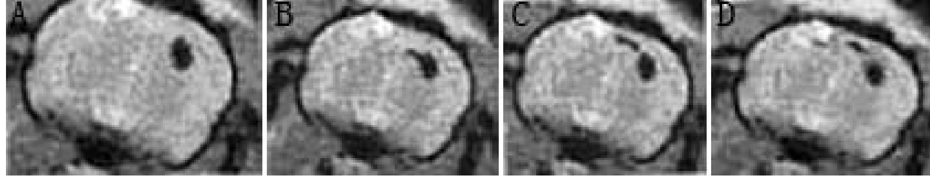

Figure 3. Serial in vivo tracking of transhemispheric stem cell migration. One day after transplantation of NSCs, a hypointensity signal can be detected at injection tract (A). Over the following 21 days post-grafting in the same animal, transplanted cells migrated along the corpus callosum to reach the site of damage (B:7 days after transplantation; C: 14 days after transplantation; D: 21 days after transplantation). This indicates that migration of stem cells to the contralateral hemisphere mainly relies on transcallosal migration.

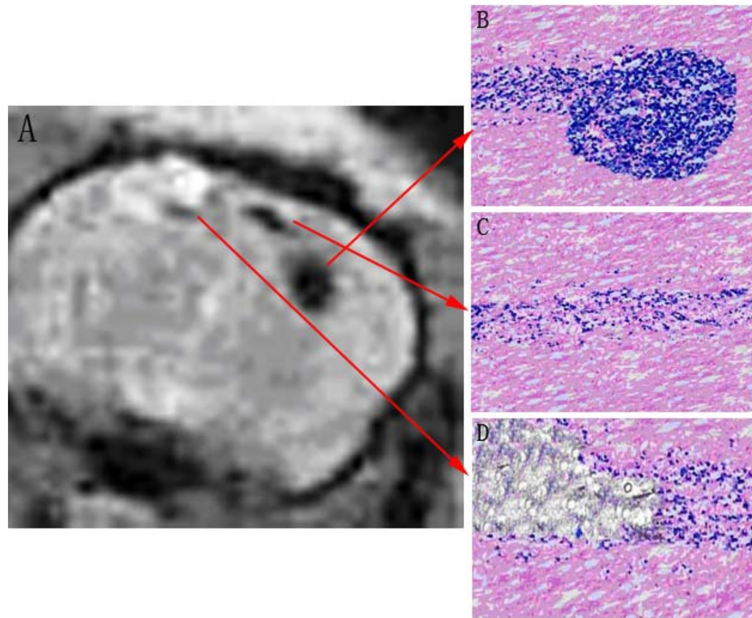

Figure 4. Histological corroboration of in vivo observations. At 21 days, Prussian blue staining showed SPIO labeled NSCs migrated from injection tract (B), crossed corpus callosum (C), and infiltrated into the lesion (D), corresponding to the planes of the MRI investigation (A).

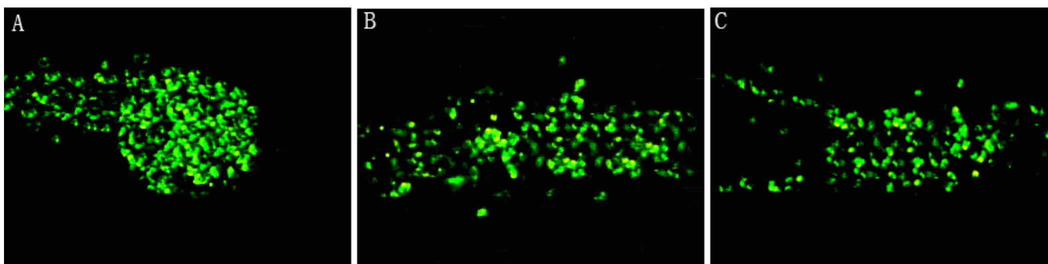

Figure 5. Fluorescent photographs showed that GFP labeled cells migrated from injection site (A), crossed corpus callosum (B), and infiltrated into the lesion (C), which was consistent with Prussian blue positive cells (B),(C) and (D) in the figure 2.

References

1. Zhu J, Zhou L, Zhang L, Zhu W, Pang L, Wang Y. Isolation and cloning of human neural stem cells along with intracerebral grafting and transgene expression in mice. Chinese J. Nervous & Mental Diseases. 2001,27 183-185.