Regenerated Host Axons Form Synapses with Neurons Derived from Neural Stem Cells Transplanted into Peripheral Nerves

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It is reported that neural stem cells (NSC) can arrest denervated muscle atrophy and promote nerve regeneration when transplanted into injured peripheral nerves, and that regenerated host axons can form synapses with transplanted and differentiated NSC. In this study, F344 rat nerve segments and F344 rat NSC were transplanted into host green fluorescence protein (GFP) transgenic F344 rats. This allowed transplanted F344 rat tissue to be used as a non-luminous background for the clear visualization of regenerated host GFP axons. Regenerated host axons grew into the transplanted F344 nerve segment 2 weeks after nerve anastomosis. Immunohistochemical staining and confocal microscope analysis revealed that regenerated host axons formed synapses with NSC-derived neurons. The findings confirmed that regenerated peripheral axons form synapses with neurons in peripheral nerves, possibly forming the basis for clinical application in peripheral nerve injury.

KEY WORDS: PERIPHERAL NERVE INJURY; NEURAL STEM CELL; SYNAPSE, ANIMAL MODEL

Introduction
Peripheral nerve injury always results in progressive muscle atrophy.1 Neural stem cells (NSC) were isolated by Reynolds et al.2 in 1992, and were found to be self-renewing and have multipotent qualities, which suggested that they may be useful in repairing central or peripheral nerve injury.3,4 Studies have verified that NSC can differentiate into neural cells, form synapses with target organs and integrate into neural circuitry when transplanted into the central nervous system.5,6 Transplanted NSC have been shown to differentiate into motor neurons and functionally innervate muscles after peripheral nerve injury,7 - 10 although the relationship between regenerated axons and transplanted NSC remains to be elucidated.

The co-existence of transplanted, differentiated NSC and other cells in repaired peripheral nerves creates a challenge in distinguishing host axons from transplanted cells, as well as in examining the precise spatial relationship between them. In the present study, F344 rat-derived nerve
segments and NSC were transplanted into green fluorescent protein (GFP) transgenic F344 host rats (GFP/F344 rats), in order to investigate whether regenerated host axons were able to form synapses with differentiated NSC.

Materials and methods
ANIMALS
Transgenic adult male GFP/F344 host rats (14 rats, mean ± SD weight 120 ± 10 g), kindly provided by Dr T Ochiya (Section for Studies on Metastasis, National Cancer Center Research Institute, Tokyo, Japan), were used as recipients.11 Pregnant non-GFP F344 rats were purchased from the Chinese Academy of Science (Shanghai, China). All experimental procedures were performed in accordance with National Institutes of Health guidelines for the care and use of laboratory animals12 and approved by the Animal Research Committee, Fudan University, Shanghai, China.

NEURAL STEM CELL PREPARATION
Rat fetuses were harvested from the pregnant F344 rats at 10 – 12 days’ gestation. Spinal cords were harvested from non-GFP F344 fetuses and prepared as described previously.10 NSC isolated from fetal spinal cords were incubated at a density of 2 x 10^5 cells/ml in Dulbecco’s Modified Eagle’s Medium (DMEM)/F12 supplemented with epidermal growth factor (20 ng/ml, R&D Systems Inc., Minneapolis, MN, USA), recombinant human basic fibroblast growth factor (20 ng/ml, R&D Systems), N2 supplement (10 ng/ml, Invitrogen, Carlsbad, CA, USA) and B27 supplement (20 ng/ml, Invitrogen).2,13

The NSC harvested from F344 fetal rat spinal cords were passaged once a week. To determine their readiness for transplantation, after two to four generations the NSC were tested for the presence of nestin protein using immunocytochemistry. Cells were fixed with 4% paraformaldehyde for 10 min, washed three times with 0.1 M phosphate-buffered saline (PBS), pH 7.4, and blocked with 2% normal goat serum (v/v) in 0.3% (v/v) Triton™ X-100 in 0.1 M PBS for 1 h at room temperature. Cells were then incubated with a rabbit antirat nestin monoclonal antibody (1:1000 dilution; BD Biosciences Pharmingen, Mississauga, Ontario, Canada) for 60 min at 4°C. Excess antibody was removed by three 15 min washes in 0.5% Triton™ X-100 in 0.1 M PBS, then the cells were incubated with cyanine 3 (cy3)-conjugated goat antirabbit secondary antibody (1:100 dilution; Sigma-Aldrich, St Louis, MI, USA) for 2 h at room temperature. The cells were then washed and examined for nestin positivity by fluorescence microscopy (Leica DC500; Leica Microsystems Imaging Solutions Ltd, Cambridge, UK).6,10

ESTABLISHMENT OF TRANSPLANTATION MODEL AND EXPERIMENTAL DESIGN
The male GFP/F344 rats were divided randomly into NSC-transplanted and control groups (n = 7 each) and anaesthetized via intraperitoneal injection of 10% chloral hydrate (40 mg/kg, Wuhan Kanglong Century Co. Ltd, Wuhan, China). The right sciatic, tibial and common peroneal nerves were exposed through a midline incision, and a 10-mm long tibial nerve segment proximal to the knee was resected. The tibial nerve stump was ligated and sewn into muscles to prevent re-innervation, and a 15 mm nerve segment obtained from non-GFP F344 fetuses was sutured to the distal tibial nerve. Then, 5 µl of culture medium containing 5 x 10^6 F344 NSC was injected into the transplanted F344
nerve segment in the NSC-transplanted group; the control group was injected with 5 µl of culture medium alone. The proximal end of the transplanted F344 nerve was ligated in order to avoid NSC leakage in both the NSC-transplanted and control groups. The proximal tibial nerve and transplanted F344 nerve were exposed and sutured together 2 weeks after the initial surgery, and the nerve segments were harvested after a further 2 weeks. The number of host GFP axons was calculated using laser scanning confocal microscopy (Leica DC500; Leica Microsystems Imaging Solutions Ltd), and the pass rate was expressed as the axon number in the transplanted nerve segment divided by that in the host axon. Fig. 1 provides a diagrammatic representation of the surgery performed.

**IMMUNOHISTOCHEMICAL ANALYSIS OF NSC DIFFERENTIATION AND SYNAPSE FORMATION**

Harvested nerves were fixed in 4% paraformaldehyde in 0.1 M PBS, pH 7.4, for 24 h, and serially treated in 0.05 M Tris-buffered saline containing 10%, 20% and 30% sucrose.

**FIGURE 1:** Animal model design. (A) A 15-mm F344 rat nerve segment was transplanted into each host green fluorescent protein (GFP) rat. Culture medium (5 µl) containing $5 \times 10^6$ F344 neural stem cells (NSC) was injected into the transplanted F344 nerve segment in the NSC-transplanted group; the control group was injected with 5 µl of culture medium alone. The proximal nerve end was ligated. The proximal tibial nerve and transplanted F344 nerve were exposed and sutured together 2 weeks after the initial surgery, and the nerve segments were harvested after a further 2 weeks. The number of host GFP axons was calculated using laser scanning confocal microscopy (Leica DC500; Leica Microsystems Imaging Solutions Ltd), and the pass rate was expressed as the axon number in the transplanted nerve segment divided by that in the host axon. Fig. 1 provides a diagrammatic representation of the surgery performed.
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(24 h for each procedure). The nerve tissues were then embedded in OCT Compound (Tissue-Tek®; Ted Pella Inc., Redding, CA, USA) and sectioned longitudinally (20 µm) at −20 °C, as described elsewhere. The slides were dried at 37 °C for 12 h, blocked in dilution buffer (500 mM saline, 0.01 M PBS, 3% bovine serum albumin, 5% goat serum) for 1 h at room temperature, then incubated overnight at 4 °C with the following primary antibodies: rabbit antirat β-tubulin III polyclonal antibody (1:1000 dilution; Sigma-Aldrich) was used to identify neurons; rabbit antirat neural-glial antigen 2 (NG2) antibody (1:1000 dilution; Sigma-Aldrich) was used to identify oligodendrocytes; and goat antirat synaptophysin antibody (1:1000 dilution; Zymed Laboratories, San Francisco, CA, USA) was used to identify synapses. After three 15 min washes in 0.5% Triton™ X-100 in 0.1 M PBS, the samples were incubated with appropriate secondary antibodies (fluorescein isothiocyanate-conjugated goat antirat, cy3-conjugated goat antirabbit, and cy3-conjugated rabbit antigoat secondary antibodies; all at 1:100 dilution; Sigma-Aldrich) for 2 h at room temperature. The slides were washed three times in 0.5% Triton™ X-100 in PBS, and nuclei were counter-stained with Hoechst 33342 (1:1000 dilution; Sigma-Aldrich), which specifically stains adenine/thymine-rich regions of double-stranded DNA. Finally, the slides were mounted in glycerol and images were acquired using fluorescence microscopy and laser scanning confocal microscopy (Leica DC500; Leica Microsystems Imaging Solutions Ltd). Regenerated axons from host rats were identified by the green fluorescence of GFP under ultraviolet light.

Results

MODEL DESIGN AND APPLICATION
In this novel model, the transplanted non-GFP F344 rat nerve segments served as a non-luminous background, allowing the clear visualization of regenerated GFP/F344 transgenic host axons (Fig. 2A). NSC harvested from F344 fetal rat spinal cords were stained using antinestin monoclonal antibody after two to four generations and found to be nestin positive (Fig. 2B, 2C). At the end of the 4-week study period, host axons grew into the transplanted F344 nerve segments in both the NSC-transplanted and control groups (Fig. 3). There was no significant difference between the mean amount of regenerated axons in the NSC-transplanted group (81.3%) and the control group (80.6%).

TRANSPLANTED NSC IN PERIPHERAL NERVES
At 4 weeks after NSC transplantation, nestin staining was positive in all of the NSC-transplanted rats (Fig. 4A). Moreover, immunohistochemistry revealed the presence of other neural cells including neurons (anti-β-tubulin III, Fig. 4B) and oligodendrocytes (anti-NG2, Fig. 4C) in the NSC-transplanted group. Samples from the control group were negative for all these markers.

REGENERATED AXONS IN TRANSPLANTED NERVE SEGMENTS
Confocal microscopy revealed the presence of regenerated host axons in transplanted nerve segments (Fig. 5A). Both regenerated axons and those differentiated from NSC were stained with the neuron-specific antibody, anti-β-tubulin III (Fig. 5B). When these images were viewed side by side, it became possible to distinguish host axons from differentiated neurons (Fig. 5A, 5B; arrows). A high level of synaptophysin-specific antibody staining was present around – and closely linked to – the regenerated axons (Fig. 5C). As the observed images were made at the same
position and magnification using confocal microscopy, it was possible to overlay the three fluorescent images and demonstrate that regenerated host axons could form synapses with transplanted and differentiated neurons (Fig. 5D, 5E). In the control group, a large number of regenerated axons were observed growing into F344 nerve segments, but there was no NSC, neuronal or other neural cell-specific staining present (data not shown).

Discussion

In 1992, Reynolds et al.\(^2,13\) isolated NSC for the first time. Later studies have demonstrated that NSC transplanted into the central nervous system survive and differentiate into neural cells and also form synapses with target organs and integrate into neural circuitry.\(^5,16 - 20\) NSC have been transplanted into peripheral nerves in order to arrest denervated muscle atrophy,\(^21,22\) potentially restoring voluntary movement.\(^10\)

The relationship between regenerated axons and transplanted/differentiated NSC has not previously been studied, partly because of the difficulty in distinguishing regenerated host axons from neighbouring tissues and transplanted/differentiated NSC. The present study describes a model where

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**FIGURE 2:** Neural stem cell (NSC) preparation. (A) Viewed under ultraviolet light with a fluorescence light microscope, the green fluorescent protein (GFP)/F344 transgenic rat embryo fluoresced, but the F344 rat embryo was non-fluorescent. (B) F344 NSC were isolated from spinal cords of 10–12 day gestation rat embryos and the neurosphere formed after two to four generations in cell culture. (C) Cells of the neurosphere were nestin-positive, as shown by immunohistochemistry (scale bar 40 µm).
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the transplanted F344 rat nerve segment served as a non-luminous background for the regenerated GFP/F344 transgenic rat axons. The advantages of this model include: (i) both the control and NSC-transplanted groups were homologous, with

FIGURE 3: Regenerated axons grew into the transplanted nerve segment in (A) neural stem cell (NSC)-transplanted and (B) control rats. Images of regenerated host axons 2 weeks after nerve anastomosis showed that host axons (1) grew through the suture site (2) and into the transplanted F344 nerve segment (3). (C, D) High-magnification images of the rectangular insets show fluorescent regenerated axons in transplanted F344 nerves of both NSC-transplanted and control groups. Scale bars 20 µm

FIGURE 4: Neural stem cell differentiation 4 weeks after transplantation in a rat model, as shown by immunohistochemistry: (A) nestin-positive cells indicated the presence of neural stem cells in the transplanted nerve segment; (B) β-tubulin III (β-tub III); and (C) neural-glial antigen 2 (NG2) staining demonstrated the presence of neurons and oligodendrocytes, respectively. Cells were counterstained with Hoechst 33342 to identify cell nuclei
no requirement for immunosuppression and no possible interference of drugs on experimental results;\(^{11}\) (ii) GFP axons and F344 nerve segments were a natural visual contrast, reducing the amount of immunofluorescent staining required. Transplanted NSC could also alter the local micro-environment, possibly secreting neurotrophic factors and promoting axon regeneration.\(^{3,4,23,24}\)

In this model, transplanted NSC remained nestin-positive even 4 weeks post-surgery, indicating the presence of viable NSC at the site of transplantation. Consistent with previous studies,\(^{6,10,22,25}\) the presence of neurons and oligodendrocytes in the treatment group, compared with their absence in the control group, confirmed that neural stem cells could survive and differentiate into neural cells in peripheral nerves.

A large number of host axons had grown

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**FIGURE 5:** In a rat model, host axons formed synapses with neurons differentiated from neural stem cells. (A) Regenerated green fluorescent protein (GFP) axons were clearly visible with laser scanning confocal microscopy and blue excitation light 2 weeks after anastamosis (arrow). (B) Anti-β-tubulin III (β-tub III) antibody labelling revealed both regenerated axons (upper arrow) and GFP-negative neurons differentiated from transplanted neural stem cells (lower arrow). (C) Antisynaptophysin antibody labelling of the same field. (D) The merged image of GFP, β-tubulin III and synaptophysin shows that regenerated axons formed synapses with the differentiated neuron. (E) Diagrammatic representation of the relationship. Scale bars 10 µm
into the transplanted nerve 2 weeks after anastomosis. In normal peripheral nerves, axons terminate directly on muscle. In this model, host axons formed synapses with transplanted/differentiated NSC and original peripheral neurons may have become interneurons. Confocal microscopy revealed regenerated axons that had grown into the transplanted F344 nerve segments and formed synapses with neurons. In the control group, a large number of regenerated axons grew into the transplanted nerve segment, but no NSC, neurons or other neural cells were found.

This study confirmed that host axons are able to form synapses with neurons derived from NSC that have been transplanted into damaged peripheral nerves. Further investigation is required to determine the longevity and functionality of these synapses.

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Conflicts of interest
The authors had no conflicts of interest to declare in relation to this article.

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