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Pluripotent Hair Follicle Neural Crest Stem-Cell-Derived Neurons and Schwann Cells Functionally Repair Sciatic Nerves in Rats

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Abstract In this paper, we constructed a novel acellular nerve xenograft (ANX) seeded with neurons and Schwann cells to bridge long-distance gaps in rat sciatic nerves. The neurons and Schwann cells were induced from Sprague Dawley (SD) rat hair follicle neural crest stem cells with sonic hedgehog/retinoic acid and neuregulin 1, respectively. Fifty male SD rats were randomly divided into two groups (n=25): ANX + cells group and ANX group. A 4-cm-long sciatic nerve defect was created on the right hind limb and bridged with cell-seeded ANX in ANX + cells group or ANX alone in ANX group. We found that the implanted neurons and Schwann cells could survive by 4 weeks and as far as 52 weeks posttransplantation. In implanted grafts, chemical synaptic structures were also found under transmission electron microscope and confirmed with immunostaining of synapsin 1, a synaptic marker. The number of regenerated axons in ANX + cells group was higher than that in ANX group (P<0.01). This novel implantation of neurons and Schwann cells via acellular nerve graft may provide an alternative way for repairing peripheral nerve defect.

Keywords Neural crest stem cells · Transplantation · Regeneration · Peripheral nerves

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Introduction

Traumatic peripheral nerve defects are common in clinic conditions, but there are now no sufficiently satisfying methods for bridging nerve gaps, especially long-distance nerve gaps. Autologous nerve transplantation can restore nerve function quite well by reconnecting both stumps of severed peripheral nerve. But this mostly practiced treatment can cause local function loss of donor nerves, which results in a dilemmatic situation. Great efforts have been devoted to researches on new prospective methods for repairing peripheral nerve injury, such as transplantation of Schwann cells or stem cells, implication of neurotrophic factors, and implantation of tissue-engineered nerves [1–3]. Among these methods, tissue-engineered nerve grafts seeded with cells, especially neurons and Schwann cells, have shown great promise in bridging peripheral nerve gaps and promoting axonal regeneration.

Thomas et al. [4] transplanted rat-embryo-derived neurons into the damaged tibial nerve and found that these neurons could survive in vivo and make functional connections with muscles. As an alternative source of neurons, hair follicle neural crest stem cells (NCSCs) naturally express nestin and exhibit characteristics of neural progenitor cells [5]. It has been demonstrated that transplanted hair follicle NCSCs could differentiate into neurons and Schwann cells and promote nerve regeneration both in central and peripheral nervous system [5–8]. Besides neurons, Schwann cells also play an important role in peripheral nerve regeneration. Schwann cells can help promote axonal regeneration by providing bioactive substrates on which axons migrate and by releasing molecules that regulate axonal outgrowth [9, 10].

Based on these findings, we hypothesized that neurons and Schwann cells seeded together into an acellular nerve graft for bridging the nerve gap might interact with each other and promote axonal regeneration. In this study, Sprague Dawley (SD) rat hair follicle NCSCs were induced into neurons and Schwann cells, which were then seeded into acellular nerve xenografts (ANXs) at a 1:1 ratio. Cell-seeded ANXs or ANXs alone were transplanted to bridge a 4-cm-long sciatic nerve defect. At 52 weeks posttransplantation, we examined: (1) survival and function of the transplanted neurons and Schwann cells and (2) axonal regeneration in the ANXs.

Materials and Methods

Induction of NCSCs

Bulges of hair follicles were obtained from adult SD rats according to the method described by Sieber-Blum, M. et al. [11]. The bulge explants were cultured in primary medium of DMEM/F12 (Gibco) supplemented with 10% fetal bovine serum (Gibco), 1% N2 (Gibco), 2% B27 (Gibco), 200 mM L-glutamine (Gibco), 0.25% ITS+3 (Sigma), 20 ng/ml EGF (Invitrogen) and 20 ng/ml basic fibroblast growth factor (Invitrogen). Four days after onset of NCSCs emigration, the bulges and culture medium were removed, and the adhering cells were digested with 0.25% trypsin. After centrifugation, the cell pellet was resuspended in serum-free primary medium and transferred into a collagen-coated six-well plate, adjusted to 1×10^4 cells per well. Sonic hedgehog (Shh)/retinoic acid (RA; Shh, 500 ng/ml, R&D; RA, 2000 nM, Sigma) was added into the medium for induction into neurons and neuregulin 1 (NRG-1; 125 ng/ml, Prospec-Tany Techno Gene) for induction into Schwann cells. The induction lasted 7 days with differentiation medium replenished every 3 days.

Characterization and Labeling of NCSC-Derived Cells

The induced neurons or Schwann cells were trypsinized into suspension, transferred into a 12-well plate, adjusted to 5,000 cells per well, and cultured with 12-mm round collagen-coated coverslips overnight. The coverslips were taken out and rinsed three times shortly with 0.01 M phosphate-buffered saline (PBS). The cells were fixed with 4% paraformaldehyde in PBS, washed, permeabilized with 0.25% Triton-X 100, and blocked with 10% normal goat serum at 37°C for 30 min. Cells were incubated at 4°C overnight with primary antibodies against Sox10 (monoclonal rabbit antibody, 1:200, Sigma), nestin (monoclonal mouse antibody, 1:50, R&D), CD34 (polyclonal rabbit antibody, 1:200, Boster), CK19 (polyclonal rabbit antibody, 1:200, Boster), β-III tubulin (Tuj1, monoclonal mouse antibody, 1:50, R&D), and S100 (monoclonal rabbit antibody, 1:500, Dako), respectively. Secondary antibodies (fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) goat antirabbit and antimouse IgG, 1: 200, Sigma) were applied at room temperature for 30 min. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/ml, Sigma) at 37°C for 15 min. Pictures were obtained with an inverse fluorescence microscope (IX70, Olympus).

For tracing seeded cells in vivo, the enhanced green fluorescent protein (EGFP) gene was stably transfected into the induced cells with a recombinant adeno-associated virus serotype 2 (rAAV2) vector at a multiplicity of infection of 100,000 virus particles per cell (rAAV2/EGFP kit, Vector Gene Technology).

Preparation of ANXs

Beagle sciatic nerves were rapidly harvested from acutely sacrificed animals of another experiment which had no effect on nervous tissues. These nerves were cut into 4-cm segments, washed with calcium- and magnesium-free PBS (CMF-PBS), and incubated with 1% lysolecithin in 0.01 M PBS at room temperature for 4 days. For the last 2 days, the solution was added with 10 mM $CaCl_2$. The incubation solution was changed everyday. The nerve segments were washed and incubated in CMF-PBS supplemented with 600 U/ml DNase I and 10 U/ml RNase A at 37°C for 24 h. After washing with CMF-PBS three times for 20 min each, the nerves were lyophilized, irradiated with Co60 gamma rays, and stored at −80°C. The acellular nerve xenografts were examined microscopically with hematoxylin–eosin (HE) staining, HLA-1 immunostaining, and scanning electron microscope.

Cell Seeding Procedure

Fifty adult male SD rats, weighing 180–200 g, provided by the Animal Center of Second Military Medical University (SMMU), were randomly divided into two groups with 25 animals in each group: ANX + cells group and ANX group. We appropriately minimized the number of animals and their suffering. All surgical procedures were compliant with the animal welfare guidelines of the ethical committee of SMMU.

After stable expression of EGFP, the NCSC-derived neurons and Schwann cells were trypsinized, suspended in PBS, mixed at a 1:1 ratio, and adjusted to 1.5×10^6 cells per milliliter. One hundred sixty microliter cell suspension was injected into each 4-cm-long ANX using a microinjector under a stereo microscope at ×10 magnification (SXP-10, Shanghai Medical Instruments). To perform the injection, the

microinjector needle was longitudinally inserted from either end of the nerve graft, and cells were injected in 20-μl volumes at eight evenly spaced points as the injector was withdrawn [12, 13]. And all injections were very slowly processed to minimize possible cell damage caused by shear force. For ANX group, equal volume of PBS was used instead of cell suspension. These nerve grafts were immediately transplanted to bridge nerve gaps.

ANX Transplantation

In both groups, rats were anesthetized with sodium pentobarbital (200 mg/kg body weight, i.p.); then, the right sciatic nerve was exposed and a 10-mm-long nerve segment was excised. The stumps would spontaneously retract back and extend the nerve gap to about 4 cm. A cell-seeded ANX or ANX alone was anastomosed with the two stumps of sciatic nerve with 9-0 Nylon in either group. Animals were returned to standard housing in a 12-h light/dark cycle at a constant temperature of 25°C, with free access to water and food.

Immunohistochemistry

At each time point of 2 and 4 weeks posttransplantation, five rats in each group were randomly chosen for immunohistochemistry study. After the rats were deeply anesthetized with sodium pentobarbital, transplanted ANXs were harvested, fixed with 4% paraformaldehyde, dehydrated in 30% sucrose solution, embedded, and cryosectioned into 10-μm sections (CM3050, Leica). We used primary antibodies against β-III tubulin (monoclonal mouse antibody, 1:50, R&D) and S100 (monoclonal rabbit antibody, 1:500, Dako) to examine the survival of seeded neurons and Schwann cells. The secondary antibodies were FITC antirabbit IgG and TRITC antimouse IgG (1:200, Sigma) in 0.4% Triton/PBS. Pictures were obtained with an inverse fluorescence microscope (IX70, Olympus).

Horseradish Peroxidase Retrograde Tracing

Two days before 52 weeks posttransplantation, five rats in each group were randomly chosen for horseradish peroxidase (HRP) retrograde tracing and immunostaining. The right sciatic nerve was exposed under anesthetization, and 15 μl of 30% HRP solution was injected into the belly of gastrocnemius muscles with a microinjector. The needle was kept in situ for 5 min to preserve the injected HRP solution.

At 52 weeks posttransplantation, these HRP-injected animals were deeply anesthetized and transcardially perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) supplemented with 0.25% glutaraldehyde. The transplanted ANXs were harvested, postfixed in the same fixative, dehydrated in 30% sucrose at 4°C overnight, and longitudinally cryosectioned into 10-μm sections (CM3050, Leica). Sections were dehydrated and mounted onto slides for tetramethylbenzidine (TMB) staining and immunohistochemistry. Neurons labeled with HRP were observed with TMB staining according to the procedure described by Mesulam, M.M. [14].

Immunohistochemistry used monoclonal IgG primary antibodies against β-III tubulin (mouse antibody, 1:50, R&D), S100 (rabbit antibody, 1:500, Dako), neurofilament 200 (NF200; mouse antibody, 1: 100, Cell Signaling), and synapsin 1 (rabbit antibody, 1:200, Sigma). β-III tubulin, S100, and NF200 were stained with diaminobenzidine (DAB) staining with biotin-conjugated secondary antibodies of goat antimouse and antirabbit IgG (1:200, Sigma). We also stained synapsin 1 and NF200 with immunofluorescent method with secondary antibodies of FITC antirabbit IgG and TRITC antimouse IgG (1:200, Sigma) in 0.4% Triton/PBS. Pictures were obtained with an inverse fluorescence microscope (IX70, Olympus) and a laser scanning confocal microscope (TCS SP5, Leica).

TEM Observation and Toluidine Blue Staining

At 52 weeks posttransplantation, five rats in each group were randomly chosen and sacrificed. The transplanted ANXs were harvested, fixed, and cut into small pieces for TEM observation and toluidine blue staining. ANX samples were dehydrated in ascending concentration gradient of ethanols and embedded by acetone and cut into ultrathin sections, stained with uranyl acetate and observed under a transmission electron microscope (JEM-1200EX, JEOL). Cross sections with 1-μm thickness were used for toluidine blue staining and observed under a light microscope (IX70, Olympus). Pictures of toluidine-blue-stained cross-sectioned nerve fibers were analyzed with Image-Pro Plus 4.5 to measure the number of myelinated fibers per square millimeter, the average thickness of myelin sheath, and the ratio of myelinated fibers to total nerve fibers.

Electrophysiology In Vitro

At 52 weeks posttransplantation, the rest of the five rats in each group were sacrificed, and the transplanted ANXs were dissected out and rinsed with Ringer's solution. Adjacent tissues enwrapping the ANXs were cleaned. The grafts were mounted onto the Medlab bio-signal operating system (Medease Science and Technology) for electrophysiological test. Electrodes with a distance of 1 cm were used to generate single stimulus, with 10-ms duration, 2-V amplitude, and 5-ms wave width. Parameters of amplifier were set as follows: magnification 50 times, frequency of upper limit 1,000 Hz, and 1-ms time constant.

Statistical Analysis

Descriptions of continuous variables are presented as mean ± standard deviation. One-way ANOVA analysis was performed with SPSS 13.0.

Results

Characterization of NCSC-Derived Cells

NCSCs emigrating from bulge of hair follicles expanded into confluence within 3–4 days. The NCSCs were nestin and Sox10 immunopositive (Fig. 1c, d) and CD34 and CK19 immunonegative (data not shown). Immunostaining also showed that the majority of the induced neurons and Schwann cells expressed β-III tubulin (Fig. 1h) and S100 (Fig. 1g), respectively, at 7 days postinduction. After transfection with rAAV2/EGFP, about one third of the NCSCs-derived neurons and Schwann cells died, while the cells left kept good viability and stably expressed green fluorescent protein (GFP; Fig. 1i, j).

Acellular Nerve Grafts

We found no residual cells left in the acellular nerve grafts via HE staining (Fig. 2a), and they were immunonegative for HLA-1 (data not shown). The scanning electron microscope showed intact collagen structures (Fig. 2b).

In Vivo Survival of Seeded Cells

At 2 and 4 weeks posttransplantation, immunostaining showed that GFP-labeled cells expressed neuron's marker β-III tubulin and Schwann cell's marker S100 in ANX + cells group (Fig. 2c–f). There was no β-III-tubulin- or S100-positive staining found in ANX group (data not shown). HRP retrograde tracing also showed that the seeded neurons

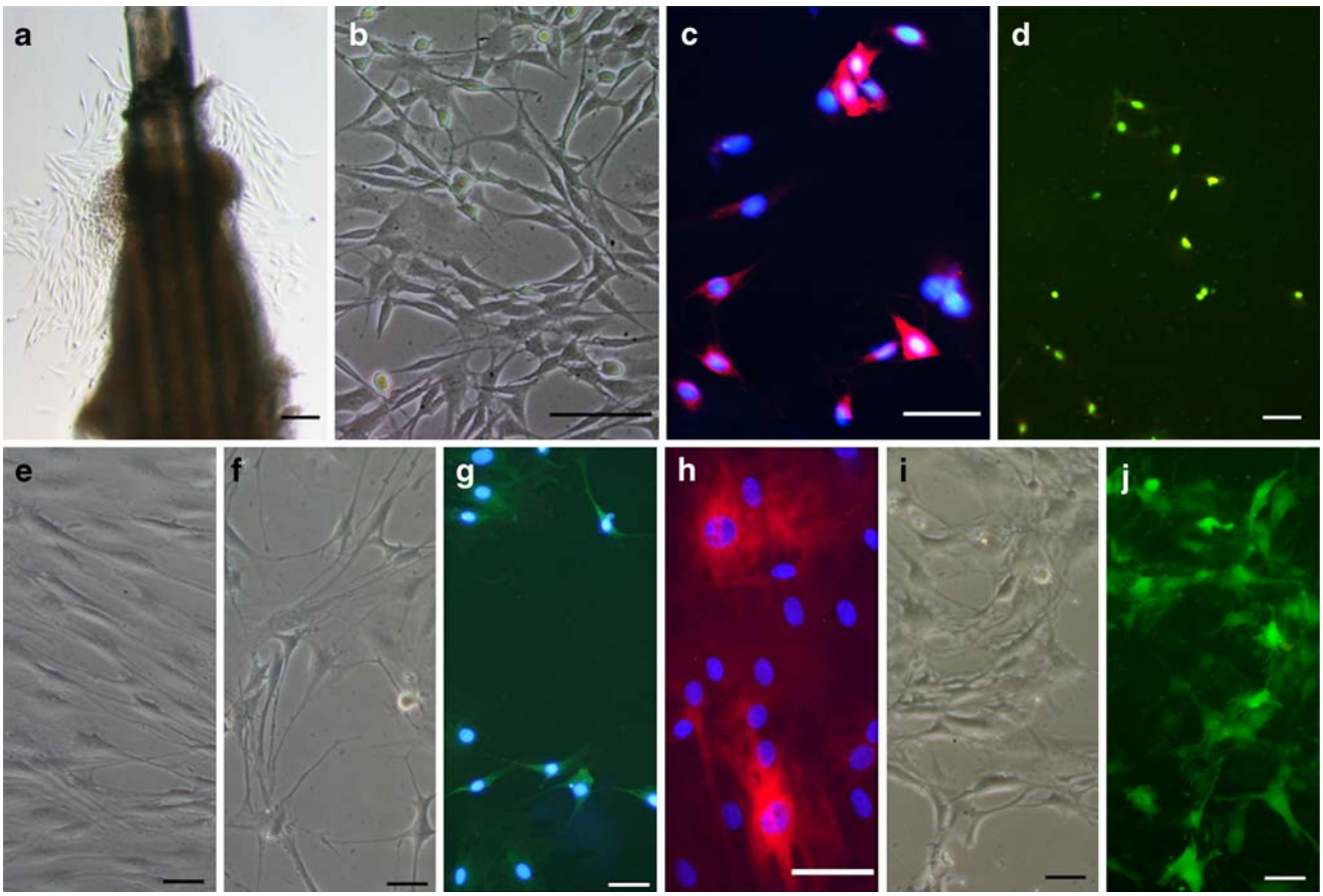

Fig. 1 Culture, characterization, induction, and transfection of hair follicle NCSCs. NCSCs migrated from the bulge of hair follicle at 4 days post explantation (**a**). Subcultured NCSCs in collagen-coated plate (**b**) were nestin positive (**c**, *red*) and Sox10 positive (**d**, *green*). After 7 days induction, NRG-1 induced NCSCs into spindle-shaped Schwann cells (**e**). Shh/RA induced NCSCs into neuron-like cells with numerous long processes (**f**). The induced Schwann cells were S100 positive (**g**, *green*), and the induced neurons were β- III tubulin-positive (**h**, *red*). Nuclei were counterstained with DAPI (**c**, **g**, **h**, *blue*). Most of the EGFP gene transfected cells (**i**) stably express GFP (**j**, *green*; **i** and **j** were the same field; *scale bar* 50 μm)

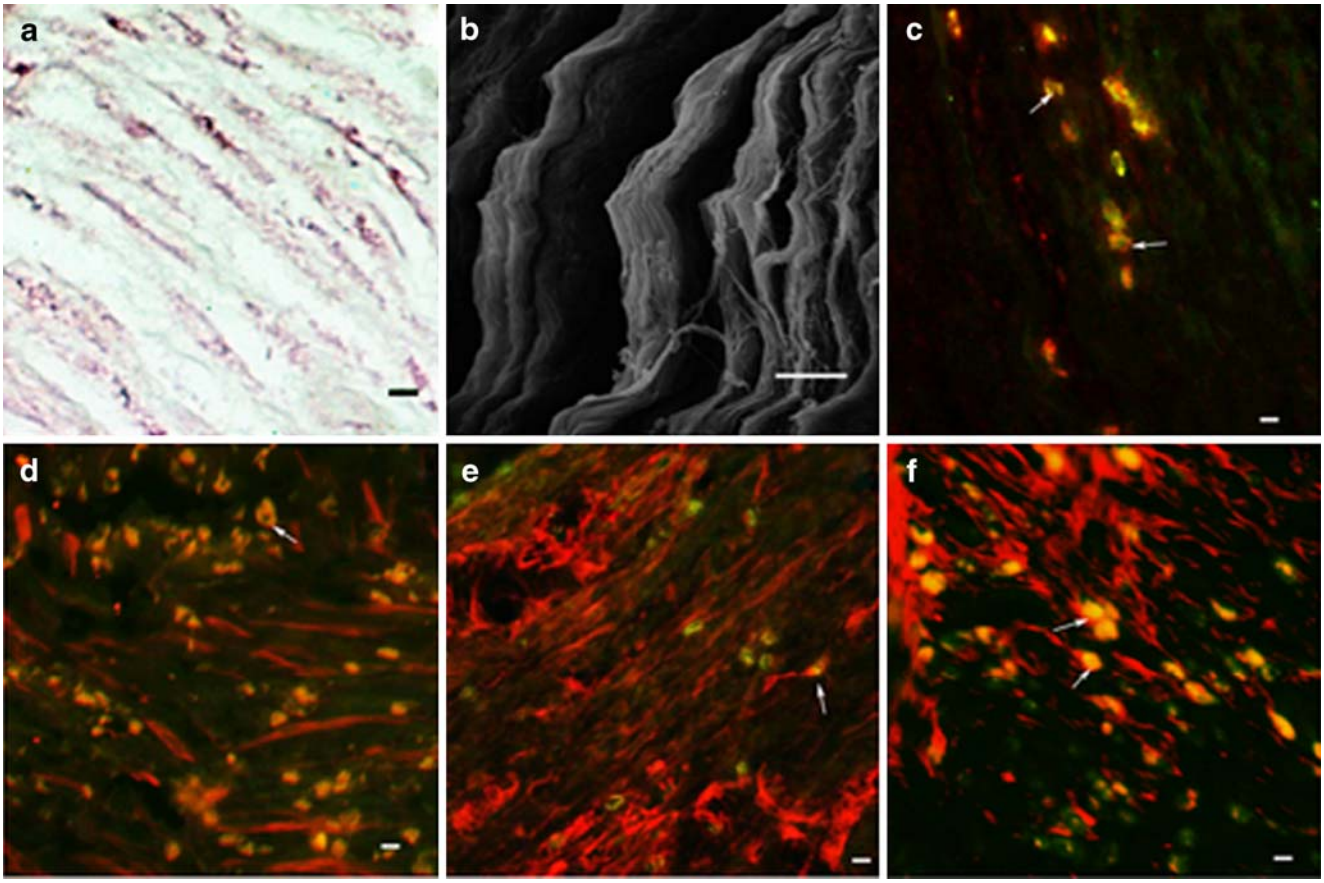

Fig. 2 After lysolecithin treatment, there was no residual cells (**a**, HE staining) left in ANXs. Scanning electron microscope showed intact structure of collagen (**b**). Coexpression (*yellow*) of EGFP (*green*) and S100 (*red*) was indicated with *arrow* (**c**, 2w; **d**, 4w). Coexpression (*yellow*) of EGFP (*green*) and β-III tubulin (*red*) was indicated with *arrow* (**e**, 2w; **f**, 4w; *scale bar* 10 μm)

survived in vivo by 52 weeks posttransplantation (Fig. 4c). At the same time point, immunoreactivity of S100, β-III tubulin, and NF200 were demonstrated with DAB staining in ANX + cells group (Figs. 3 and 4e).

Axonal Regeneration

By 52 weeks posttransplantation, chemical synaptic structures were found in nerve grafts of ANX + cells group with immunohistochemistry (Fig. 4d) and TEM (Fig. 4f). Toluidine blue staining showed regenerated axons in both groups (Fig. 4a, b). In ANX + cells group, the number of myelinated fibers per square millimeter, the average thickness of myelin sheath, and the ratio of myelinated fibers to total nerve fibers were higher than that in ANX group (Table 1).

Electrophysiology

Electrophysiology in vitro showed that the conduction velocity of nerve grafts in ANX + cells group (24.522±0.884489 m/s) was lower than that in ANX group (31.408±2.510104 m/s; P=0.000412), which might be caused by synaptic structures resulting in slower conduction in the nerve grafts.

Discussion

In this paper, we demonstrated that NCSC-derived neurons and Schwann cells, seeded in tissue-engineered ANXs bridging large nerve defects, could survive, keep long-term neuron differentiation, and promote axonal regeneration in rat sciatic nerves.

NCSCs Induction

NCSCs can be easily obtained from the bulge of hair follicles with a minimal invasive method [11, 15]. This advantage makes NCSCs quite suitable as a source for large quantity of cells like neurons and Schwann cells, which can be autologously transplanted for various purposes without immune rejection.

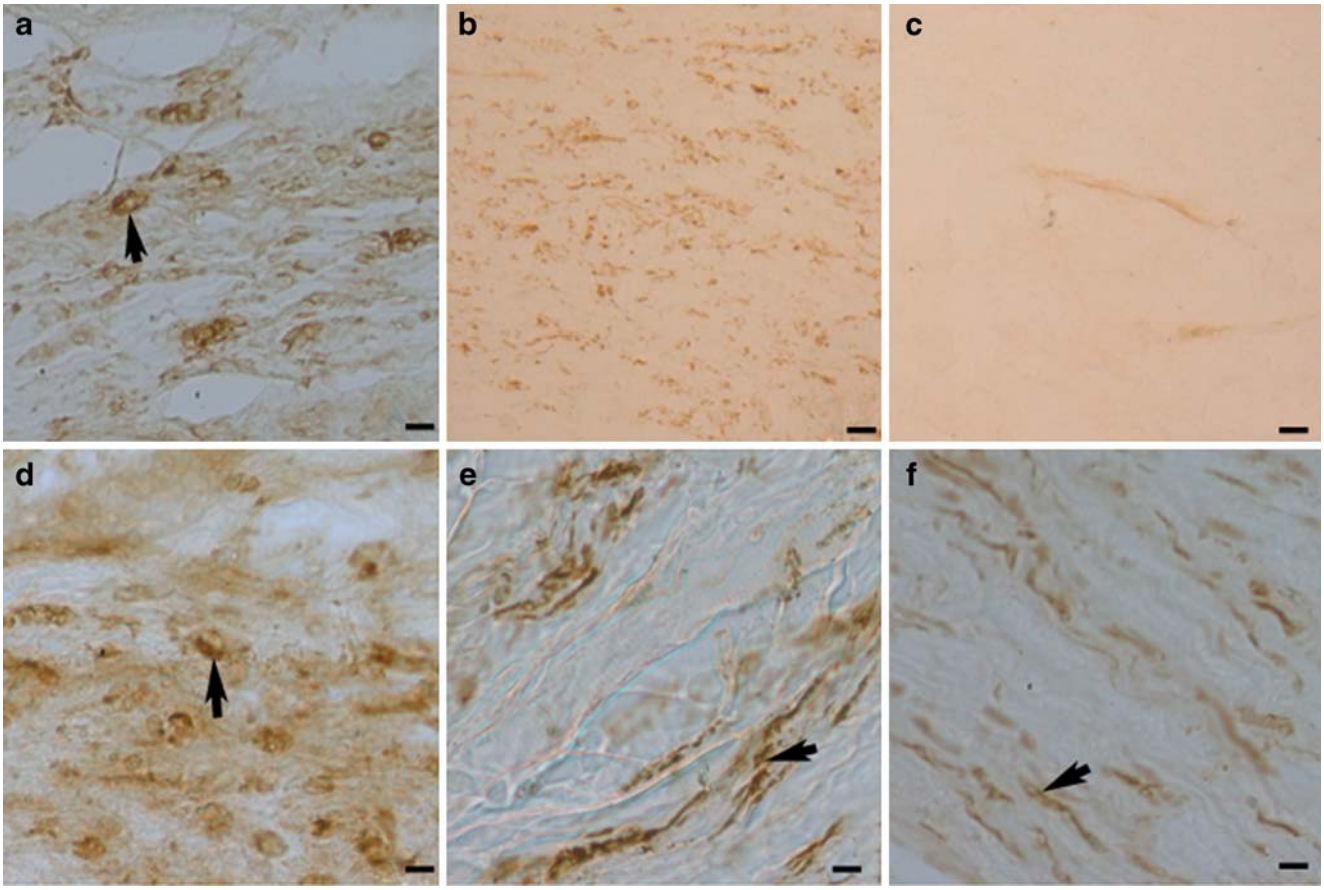

Fig. 3 Immunohistochemistry of ANXs at 52 weeks posttransplantation. **a**–**c** ANX group. **d**–**f** ANX + cells group. **a** and **d** showed S100-positive cells (*arrow*) in both groups. There were no β-III-tubulin- or NF200-positive cells in ANX group (**b**, **c**). While in ANX + cells group, β-III-tubulin- and NF200-positive cells were quite prevalent (*scale bar* 10 μm)

Shh is a soluble signaling protein which can pattern cell differentiation in neural tubes and limb buds [16–18]. RA can induce both CD133+ hematopoietic stem cells and neuroepithelial cells into neurons [19, 20]. Shh combined with RA can induce embryonic stem cells into motor neurons [21]. Similar to these findings, we induced NCSCs into β-III-tubulin-expressing neurons with Shh/RA.

In terms of Schwann cell induction, NRG-1 has successfully induced the boundary cap NCSCs into mature Schwann cells [22]. Spinal cord dorsal root ganglia derived boundary cap NCSCs and hair follicle NCSCs are both neural-crest-originated, which suggests that the latter may also respond to the induction of NRG-1. Our data supported this suggestion by inducing hair follicle NCSCs to S-100-immunopositive Schwann cells with NRG-1 [23].

The Scaffold of ANXs

In the scaffold, cell debris must be removed before nerve fibers can regenerate [24, 25]. Lysolecithin processing can dissolve cell membranes, remove myelin sheaths, and keep the intrinsic characteristics of a normal nerve matrix. ANXs mainly consist of collagen network and other glycoproteins forming the basal laminae, endoneurium, perineurium, and epineurium. The empty collagen network in ANXs is permeable to cells so that the grafts can be rapidly recolonized with seeded neurons and neurites [26].

In Vivo Survival of Seeded Cells

The microenvironment in injured sciatic nerves is favorable for the axonal regeneration [27]. This nourishing environment for nerve regeneration is created by neurotrophic factors, including nerve growth factor, ciliary neurotrophic factor, and basic fibroblast growth factor, which are released by proliferating Schwann cells residing in peripheral nerves [28–30]. In injured sciatic nerves, dorsal root ganglia neurons can survive to 4 months after transplantation [31], indicating that such a nourishing environment can support neuron survival. Our study observed the survival of hair follicle NCSC-derived neurons in ANXs in vivo by 2, 4, and 52 weeks posttransplantation. We speculate that in

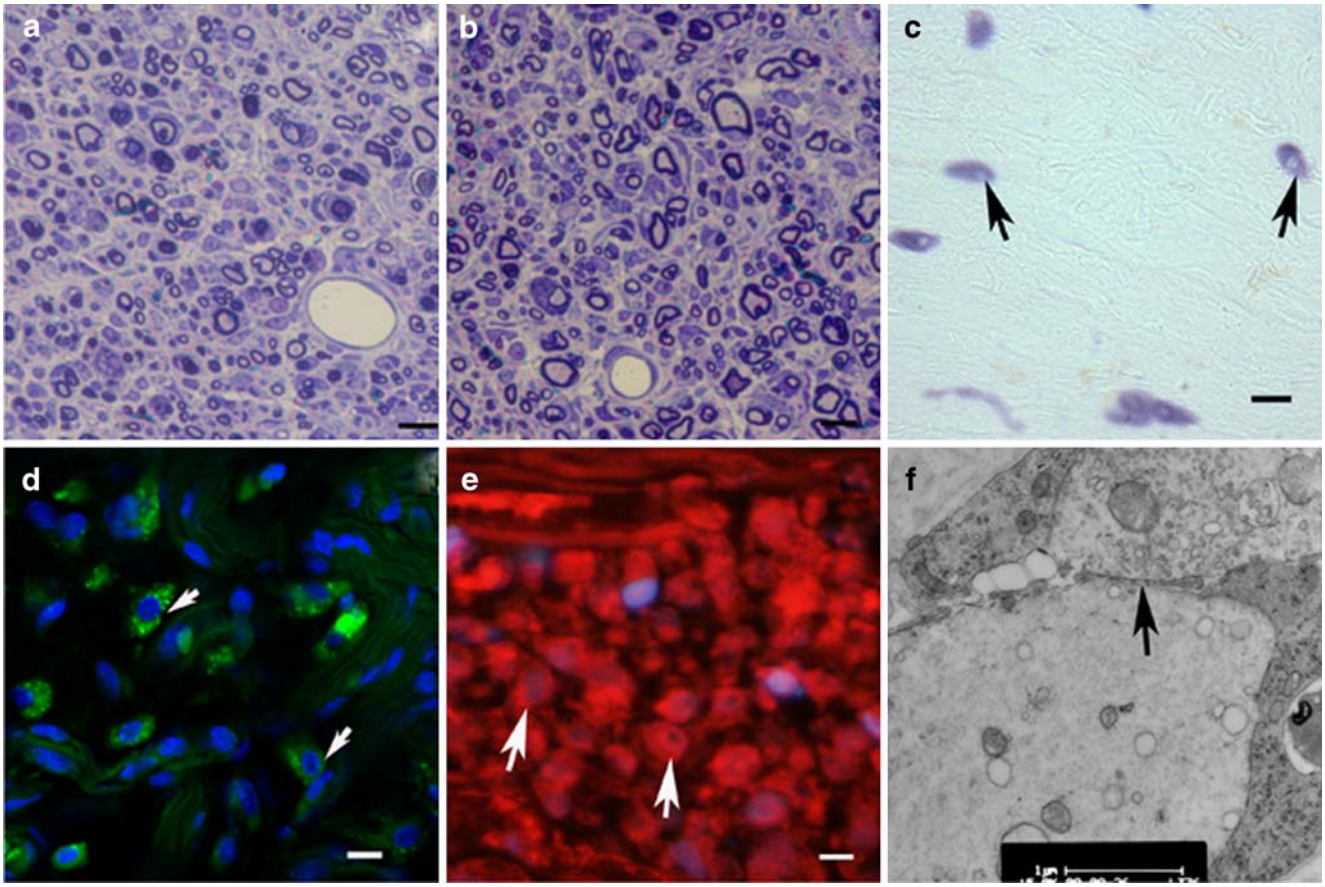

Fig. 4 ANXs harvested at 52 weeks posttransplantation. Toluidine-blue-stained regenerated axons in nerve grafts of ANX group (**a**) and ANX + cells group (**b**). HRP retrograde tracing showed seeded neurons in ANX + cells group (**c**). Expression of synapsin-1 (**d**, *green*) and NF200 (**e**, *red*) was found in ANX + cells group. Nuclei were counterstained with DAPI (**d**, **e**, *blue*). Synaptic structure in ANX + cells group was found under TEM, *arrow* showing a synaptic cleft, under which there were numerous synaptic vesicles (**f**; *scale bar* **a**–**e** 10 μm; **f** 1 μm)

the transplanted ANXs a similarly nourishing environment existed and supported the survival of neurons, possibly induced by the seeded NCSC-derived Schwann cells playing the role of native Schwann cells in injured nerves. In addition, such a nourishing environment might help maintain the long-term and stable differentiated status of the NSCS-derived neurons.

Axonal Regeneration

In cell-seeded ANXs, we found more myelinated axons per square millimeter than in non-cell-seeded ANXs. The relationship between axonal regeneration and seeded NCSC-derived neurons and Schwann cells is uncertain due to the limitation of evidence. We can safely infer that the seeded cells promoted axonal regeneration and myelination. NCSC-derived Schwann cells might be involved in the myelination directly and promote axonal regeneration by releasing neurotrophic factors that regulate axonal outgrowth [9, 10].

The formation of synaptic structures in the cell-seeded grafts indicated the possibility that the transplanted NCSC-derived neurons might play a role of "relay station" between the regenerated axons and their targets. A seeded

Table 1 Regenerated myelinated fiber analysis in both groups $(n = 5, \bar{x} \pm s)$ (toluidine blue staining)

Group	Myelinated fiber (root/mm^2)	Average thickness of myelin sheath (μm)	Myelinated fibers/total nerve fibers (%)
ANX	3,595±321	1.76±0.50	40±4.32
ANX + cells	7,238±540[a]	3.89±0.23[a]	58±3.61[a]

[a] Vs ANX group

neuron might receive stimulus from a regenerated axon and then send the signal along its own axon to the effector. The in vitro electrophysiology revealed a slower conduction velocity in cell-seeded ANXs, which might be a result of the "relaying effect" of seeded neurons. This might be helpful in shortening the absence of muscular innervation and preventing muscular atrophy.

Our future research will focus on the effect and mechanism of the seeded NCSC-derived neurons and Schwann cells on the axonal regeneration.

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