

Schwann cells originating from skin-derived precursors promote peripheral nerve regeneration in rats

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Abstract

Artificial guidance channels containing Schwann cells can promote the regeneration of injured peripheral nerve over long distances. However, primary Schwann cells are not suitable for autotransplantation. Under specific conditions, skin-derived progenitors can be induced to differentiate into Schwann cells. Therefore, adult rat dorsal skin (dermis)-derived progenitors were isolated and induced to differentiate with DMEM/F12 containing B27, neuregulin 1, and forskolin. Immunofluorescence staining and reverse transcription polymerase chain reaction (RT-PCR) confirmed that the resultant cells were indeed Schwann cells. Artificial guidance channels containing skin-derived progenitors, Schwann cells originating from skin-derived progenitors, or primary Schwann cells were used to bridge 5 mm sciatic nerve defects. Schwann cells originating from skin-derived progenitors significantly promoted sciatic nerve axonal regeneration. The significant recovery of injured rat sciatic nerve function after the transplantation of Schwann cells originating from skin-derived progenitors was confirmed by electromyogram. The therapeutic effect of Schwann cells originating from skin-derived progenitors was better than that of skin-derived progenitors. These findings indicate that Schwann cells originating from skin-derived precursors can promote peripheral nerve regeneration in rats.

Key Words: nerve regeneration; skin-derived precursors; Schwann cells; peripheral nerve injury; cell transplantation; NSFC grant; neural regeneration

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Introduction

Although the axons of peripheral nerves are capable of regeneration after transection injuries, the functional recovery of target organs is rarely satisfactory (Fawcett and Keynes, 1990; Stoll and Müller, 1999). To achieve better outcomes, the distal and proximal stumps of injured nerves should be directly reconnected without putting them under tension. However, the nerve defects are sometimes too long to be repaired by direct suturing of the nerves without causing tension or leaving a gap. Many techniques have been developed to address this problem. Compared with other approaches, autologous nerve grafting is the gold standard because it provides both a nerve scaffold and activates Schwann cells to facilitate axonal regeneration (Griffin et al., 2013; Pabari et al., 2014). However, certain complications of autologous nerve grafting are unavoidable, such as numbness, sensory abnormality, functional deficiency at the donor site, and unsatisfactory outcomes for long defects or large diameter nerves. Therefore, several alternatives have been developed, including acellular nerve allografts and synthetic or biological nerve guidance channels (Lin et al., 2013; Gu et al., 2014). Although the preliminary results when using these materials to repair short gaps after nerve injury are very promising, nerve defects with long gaps failed to regenerate when treat-

ed with a conduit alone (di Summa et al., 2011). Because cellular components, such as Schwann cells, appear indispensable during axonal regeneration of peripheral nerves, nerve conduits supplemented with extrinsic Schwann cells were proposed (Erba et al., 2010; Sun et al., 2011). Further research confirmed that artificial guidance channels seeded with Schwann cells can remarkably enhance the regeneration of peripheral nerves to repair large gap nerve defects (Evans et al., 1998, 2002; Hadlock et al., 2000; Mosahebi et al., 2001; Levi et al., 2002).

Because primary Schwann cells are not accessible for harvest and seeding into autografts, the differentiation of embryonic stem cells into Schwann cells and neural stem cells were proposed as candidates for transplantation (Cui et al., 2008; Xu et al., 2012). However, ethical concerns and immunological rejection are associated with these types of cells, which has so far limited their clinical application. Recently, a type of novel multipotent progenitor cell, skin-derived precursors (SKPs), has been reported in the dermis of both neonatal and adult skin (Toma et al., 2001). SKPs are characterized by similar gene expression patterns and functional properties as neural crest-derived cells in the embryonic stage (Toma et al., 2001). A previous study has demonstrated that, under specific differentiating culture conditions, SKPs

can be induced to differentiate into neurons and Schwann cells (Fernandes et al., 2004). McKenzie et al. (2006) reported that when SKPs-derived Schwann cells were transplanted into the peripheral nerve of Shiverer mutant mice, which are genetically deficient in the basic protein myelin, they were able to myelinate and functionally integrate into the axons of host sciatic nerve. Marchesi et al. (2007) demonstrated that synthetic and collagen-based nerve conduits seeded with SKP-derived Schwann cells could be used to repair a 16 mm gap in rat sciatic nerve. Similarly, Walsh et al. (2009) seeded SKP-SCs into acellular nerve and found that the SKP-SCs remarkably enhanced nerve regeneration in 15 mm nerve defects in rats. Together, these data suggest that SKP-SCs may be an accessible and efficacious cell source for promoting peripheral nerve regeneration. However, to the best of our knowledge, the reported analyses of the effect of SKPs or SKP-SCs on nerve regeneration are often confounded by the different types of synthetic or natural nerve conduits used, and the function of the cells is not separately or independently evaluated. To address this problem, we used silicone tubes seeded with different types of cells to determine the efficacy of SKPs and SKP-SCs for promoting nerve regeneration.

Materials and Methods

Cell culture and differentiation

We prepared cells using previously described protocols (Toma et al., 2001; Fernandes et al., 2004). Briefly, 8-week-old male Sprague-Dawley rats of clean grade were provided by the Laboratory Animal Center of Tongji Medical College, Huazhong University of Science and Technology, China (license No. SCXK (E) 2003-0001). The procedures in this study conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996), and the protocol was approved by the Institutional Animal Care Committee of Tongji Medical College, Huazhong University of Science and Technology, China. To prepare the SKPs, a group of rats was euthanized by CO₂ inhalation. The dorsal skins were carefully isolated and minced into small (around 1 mm³) pieces. The minced skin tissue was then digested with collagenase II and then mechanically dispersed into single cells with the tip of a polished Pasteur pipette. Next, the cell suspension was spun down through a 3% bovine serum albumin gradient at 190 × g to remove any cell debris. Finally, the cells were resuspended in DMEM/F12 (Invitrogen, Grand Island, NY, USA) supplemented with basic fibroblast growth factor (bFGF; 10 ng/mL, R&D Systems, Minneapolis, MN, USA), epidermal growth factor (EGF; 20 ng/mL, R&D Systems), and B27 (1:50; Invitrogen). These primary cells were seeded at 1 × 10⁶ cells/mL in a 6-well-plate, which was placed in the incubator at 37°C with 1.5% CO₂. Culture medium was replaced every 3 days.

To induce differentiation of the SKPs, the primary cell-spheres were collected and mounted on poly-ornithine pre-treated coverslips in the differentiation medium, which was composed of DMEM/F12 supplemented with 1% B27, neuregulin-1 (10 ng/mL, R&D Systems), and 4 μmol/L forskolin (Merck,

Whitehouse Station, NJ, USA). Under these conditions, the cells rapidly attached to the culture surface and migrated out of the spheres immediately after adherence. Two weeks after differentiation, the cells were analyzed using immunocytochemistry to estimate the efficiency of SC differentiation.

Estimation of the efficiency of SC differentiation by immunofluorescence staining

For immunofluorescence staining, SKP spheres were collected and fixed in 4% paraformaldehyde at room temperature for 15 minutes. The spheres were permeabilized with 0.5% saponin and incubated with primary antibodies overnight. The differentiated SKPs were fixed in 4% paraformaldehyde and permeabilized with 0.4% Triton X-100. Next, the samples were incubated with rabbit anti-rat S100 monoclonal antibody (1:200; Abcam, Cambridge, MA, USA), mouse anti-rat βIII-tubulin monoclonal antibody (1:200; Cell Signaling, Danvers, MA, USA), or mouse anti-Nestin (1:200; Abcam) overnight at room temperature. All samples were then incubated with Alexa 488-goat anti-mouse IgG (1:1,000; Molecular Probes, Eugene, OR, USA) or Alexa 555-goat anti-rabbit IgG (1:1,000; Molecular Probes) for 1 hour at room temperature. DAPI was used for nuclear counterstaining. Staining was visualized using a fluorescence microscope (Olympus, Tokyo, Japan).

Validation of the efficiency of SC differentiation by reverse transcription PCR (RT-PCR)

RT-PCR was used to validate the immunostaining results and was performed as previously described (Chen et al., 2005). Briefly, the RNA samples were diluted to 10 ng/μL, 1 ng/μL, 100 pg/μL, and 10 pg/μL before reverse transcription, and subjected to the following PCR protocol. One microliter of RT reaction solution was added to 9 μL of a PCR mixture composed of 0.1 μL of deoxy-NTPs (100 mM), 1.1 μL of PCR Gold buffer, 0.9 μL of MgCl₂ (25 mM), 0.15 μL of AmpliTaq Gold DNA polymerase (5 U/μL; Applied Biosystems, Life Technologies, Grand Island, NY, USA), and 0.1 μL of each oligonucleotide primer (100 μM; Invitrogen). For S100β cDNA amplification, the following primers were used: 5'-ATG TCC GAG CTG GAG AAG GC-3' (sense) and 5'-TCG TCC AGC GTC TCC ATC AC-3' (antisense), producing a fragment of 188 bp. The samples were subjected to 7 minutes at 95°C; 50 cycles of 10 seconds at 95°C, 15 seconds at 63°C, and 25 seconds at 72°C; and finally 7 minutes at 72°C in a GeneAmp PCR System 2400 (Applied Biosystems). An internal control was created using ribosomal protein L19 for normalization standard with the following primers: 5'-AAG AAG GTC TGG TTG GAT CCC AAT G-3' (sense) and 5'-AGG CTG TGA TAC ATA TGG CGG TCA A-3' (antisense). Those samples were subjected to 7 minutes at 95°C; 50 cycles of 15 seconds at 95°C, 20 seconds at 59°C, and 45 seconds at 72°C; and finally 7 minutes at 72°C.

Transplantation of different cell types to repair rat sciatic nerve defects

Ninety 8-week-old Sprague-Dawley male rats were randomly

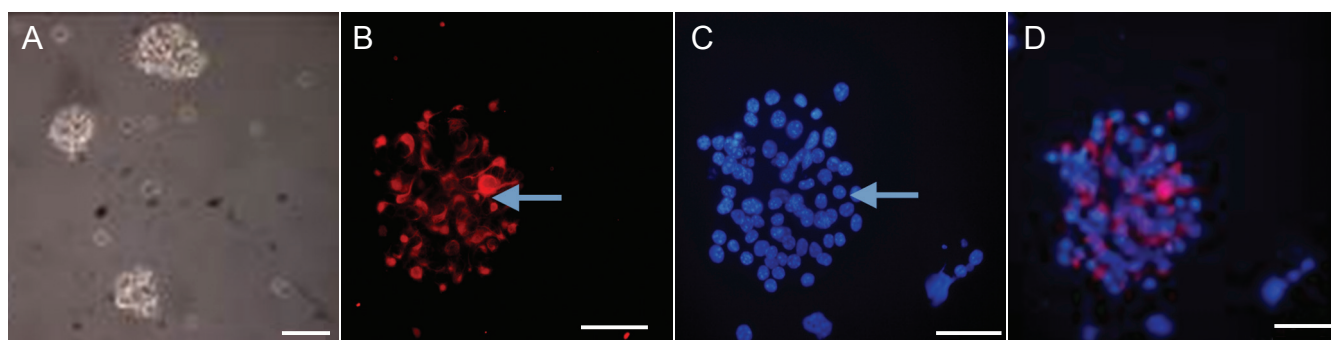


Figure 1 Isolation and culture of skin-derived progenitors (SKPs) by immunofluorescence staining.

(A) After 10 days in culture, the number and size of the SKP spheres were increased. (B) Most of the cells in the SKP spheres were nestin-positive (red; arrow). (C) The cell nuclei were counterstained with DAPI (blue; arrow). (D) Merged image of B and C. Bars: 50 μ m.

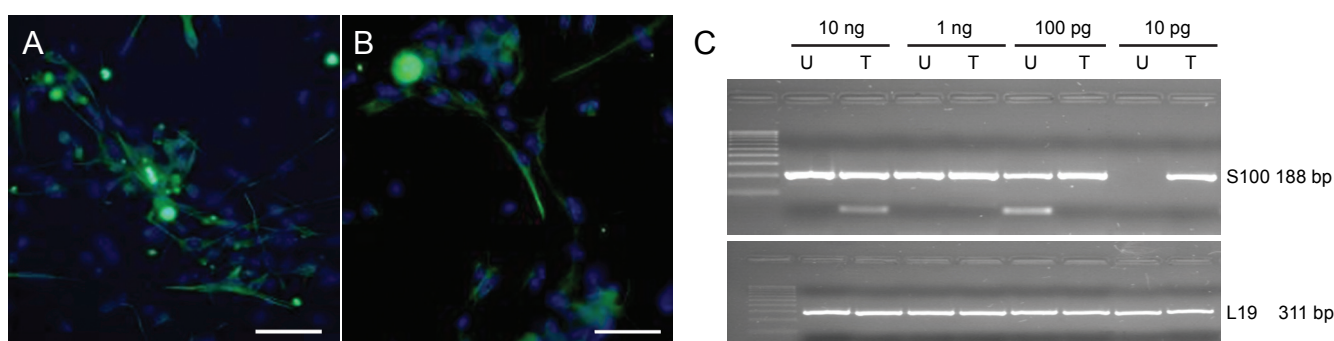


Figure 2 Schwann cell purification after differential adhesion treatment.

(A) Schwann cells originating from skin-derived progenitors (immunofluorescence staining) were passed four times. The percentage of S100 (green) cells was approximately 80%. (B) After differential adhesion, the purity of the S100-positive cells increased (immunofluorescence staining). Bars: 50 μ m. (C) Semi-quantitative PCR analysis of the expression of S100 mRNA in dilution series. 10 ng, 1 ng, 100 pg and 10 pg represented 10 ng/ μ g, 1 ng/ μ g, 100 pg/ μ g and 10 pg/ μ g of RNA sample. U: Untreated with differential adhesion; T: treated with differential adhesion.

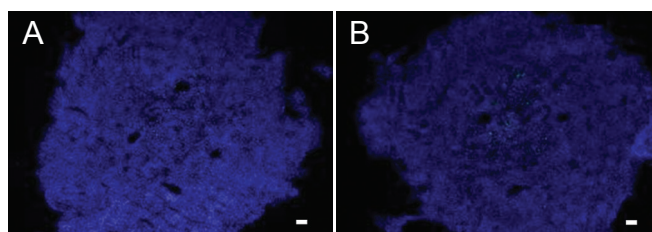


Figure 3 Transplanted green fluorescent protein (GFP)-positive cells are visible in the newly regenerated nerve (immunofluorescence staining).

(A) In the SKP group, the GFP-positive cells were visible in both the outer margins and in the center of the tissue. (B) In the SKP-SC group, the donor GFP-positive cells were only found in the center of the newly regenerated nerve. Bars: 50 μ m. SKP: Skin-derived progenitor; SKP-SC: Schwann cell originating from skin-derived progenitor.

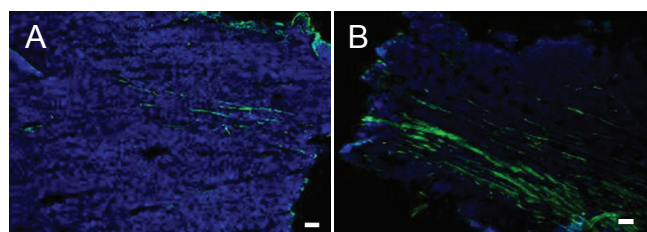


Figure 5 Schwann cells sorted in newly regenerated nerve (immunofluorescence staining).

S100-positive cells (green) in the SKP-SC group (B) were more spatially localized than in the SKP group (A). Bars: 50 μ m. SKP: Skin-derived progenitor; SKP-SC: Schwann cell originating from skin-derived progenitors.

and equally divided into three groups: SKPs, SCs, and SKP-SCs. The animals were anesthetized, and the right sciatic nerve was exposed and transected. A 5-mm-segment of the nerve in the proximal stump was excised. A custom-made silicone tube with 1 mm diameter was used to bridge the gap between the proximal and distal nerve stumps. The nerve stumps were sutured to the tube with 8-0 Prolene suture. The SKPs, SKP-SCs, and SCs were resuspended in DMEM/F12 at a density of 1×10^6 cells/mL and 15 μ L of the cell suspension was injected into each tube. Eight weeks after the operation, the bridged sciatic nerve was exposed. After removing the silicone tube, we found that the nerve gaps had been

connected by regenerated nerve tissue in all groups. To confirm these results, green fluorescent protein (GFP)-transgenic rats were provided by Prof. Hugo Vankelecom (University of Leuven, Belgium). GFP-transgenic rat derived SKPs, SKP-SCs, and SCs were also harvested and transplanted into host animals as described above.

Rat neurological function detected by electromyography (EMG)

Twelve weeks after the operation, EMG analysis (Shanghai Haishen Medical Electronic Instrumentation (NID-092), Shanghai, China) was performed. One electrode was insert-

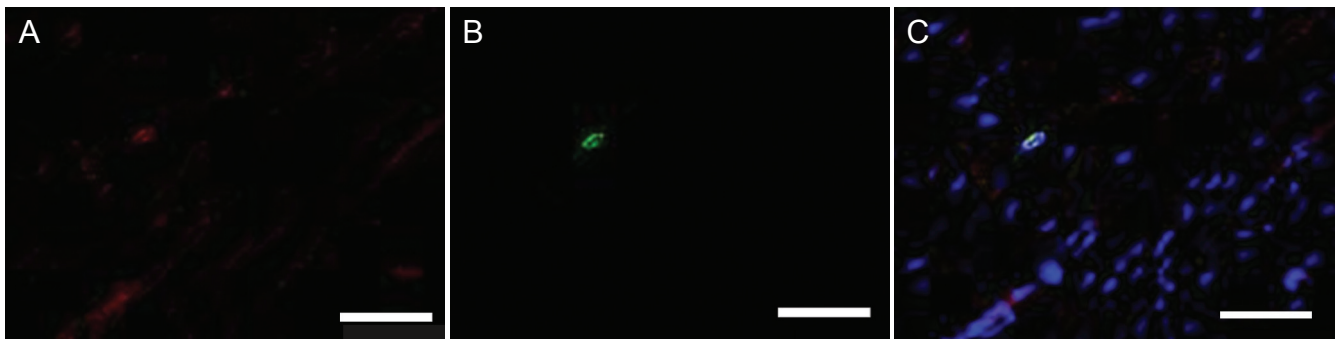


Figure 4 Transplanted green fluorescent protein (GFP)-positive cells integrated into regenerated nerve (immunofluorescence staining). Eight weeks after transplantation, the GFP-positive cells expressed S100 (red) in the host regenerated nerve. (A) S100 staining for regenerated nerve. (B) GFP-positive cells detected in the same field. (C) After DAPI staining, merged image of A and B. Bars: 50 μ m.

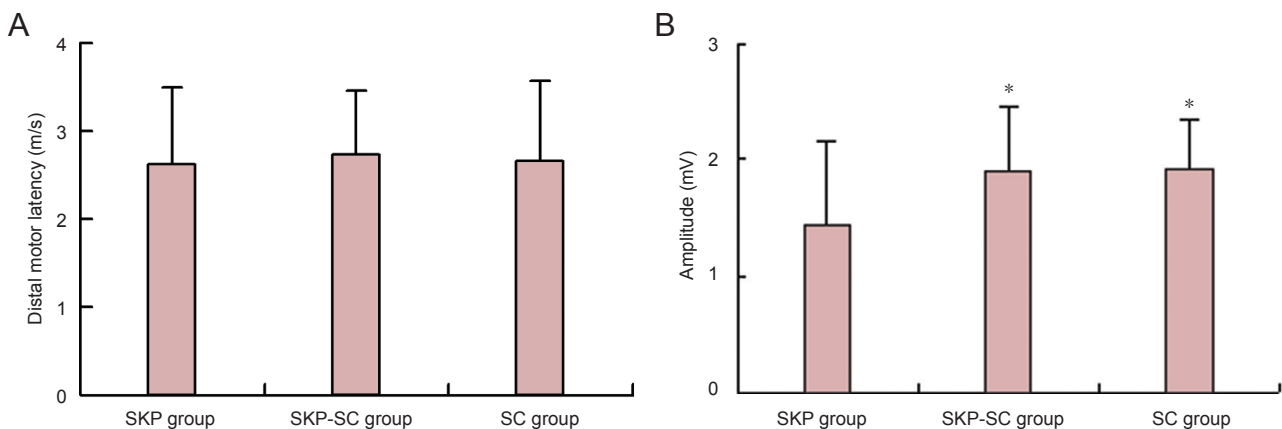


Figure 6 The electrophysiological analysis of rat sciatic nerves treated with different cells 12 weeks after transplantation. The distal motor latency (A) and amplitude data (B) for the three groups. The data were shown as mean \pm SD and were analyzed by one-way analysis of variance followed by Tukey-Kramer multiple comparison tests to determine significant differences among the groups. * $P < 0.05$, vs. SKP group. SKP: Skin-derived progenitor; SKP-SC: Schwann cell originating from skin-derived progenitor; SC: Schwann cell.

ed into the gastrocnemius muscle and the others were placed on the proximal and distal sciatic nerves at the grafting site. The distal motor latency and amplitude of the EMG signal were measured for rats from each group.

Statistical analysis

The data were expressed as mean \pm SD and were analyzed by one-way analysis of variance followed by Tukey-Kramer multiple-comparison tests to determine if there were any significant differences among the groups using NCSS 2004 statistical software (NCSS, LLC, Kaysville, UT, USA).

Results

Isolation and culture of SKPs

Primary skin dermal cells from adult rats were cultured for 3–4 days, at which point a few small non-adherent spheres were observed, but the majority of cells in culture were adhered to the surface of the plastic culture dishes. Within the following 10 days, the number of spheres and the sphere size increased. Secondary cells were also able to generate spheres in the sphere-forming medium. Further generation of spheres was obtained for more than six passages, indicating

that the cells from the SKP spheres were able to self-renew. Fourteen days later, immunofluorescence staining revealed that nearly all cells in the spheres were nestin-positive, but none of them expressed NeuN or NFM-L. These results agree with observations from previous studies (Hou et al., 2006) (Figure 1).

Differentiation and purification of SKP-SCs

To induce differentiation of the SKPs towards Schwann cells, SKPs were cultured with Schwann cell induction medium. Similar to SKP spheres in differentiation culture medium, small SKP-SC aggregates attached to the culture surface after 24 hours, and then cells around the aggregates formed processes and migrated out of the aggregates over the following 24 to 48 hours. Two weeks after induction of differentiation, the cells were stained for Schwann cell marker S100 β . At that time point, the immunocytochemistry results showed that the majority of cells in these culture conditions had begun to differentiate (70% in the Schwann cell conditions). However, the percentage of S100 β -positive cells did not increase after three passages. Therefore, we used differential adhesion to enrich the SKP-SCs in culture. We observed that the

purity of the SKP-SCs increased after differential adhesion. Semi-quantitative PCR showed that the expression of S100 β by cells after purification using differential adhesion was higher than that in untreated cells (Figure 2).

Bridging nerve defects using nerve conduits seeded with cells

Gross appearance

Eight weeks after the operation, the new segment of nerve was slightly whiter and smaller in diameter than the proximal and distal nerve stumps in all three groups. There were no significant macroscopic differences in the regenerated nerve sections among the three groups.

Schwann cell sorting and axonal regeneration

Eight weeks after the operation, the GFP-positive cells were visible in the longitudinal section of new growth nerve by fluorescence microscopy (Figure 3). Although the percentage of the total cell number that was GFP-positive was similar among the three groups, the distributions of the GFP cells were different. For example, the GFP-positive cells appeared in both ends and the center of the nerve in the longitudinal section of the SKP group, whereas in the SKP-SC and SC groups, more GFP-positive cells were found in the central area. Immunofluorescence staining showed that most of the GFP-positive cells in the SKP-SC and SC groups also expressed S100 (Figure 4). These data confirmed the survival of SKPs and SKP-SCs in the host nerve, and their integration into the axonal regeneration after grafting. Interestingly, immunofluorescence staining of the longitudinal sections of the regenerated nerve segments revealed that the S100-positive cells, including extrinsic and intrinsic cells, in the SKP-SC and SC groups were more spatially localized than in the SKP group (Figure 5). Together, these results suggest that SKP-SCs may more actively promote axonal regeneration after cell transplantation compared with naive SKPs.

Neurological function

Twelve weeks after the operation, EMG tests were performed on samples from all groups. The distal motor latency in the rat sciatic nerves of the SKP, SKP-SC, and SC groups were similar ($P > 0.05$). In contrast, the amplitude in the SKP-SC and SC groups was larger than that in the SKP group ($P < 0.05$; Figure 6).

Discussion

Peripheral nerve defects can be repaired using nerve guidance channels seeded with stem or progenitor cells. In the present study, the functions of SKPs and SKP-SCs during nerve regeneration were analyzed. Three major conclusions can be drawn from these results. First, SKP-SCs can be purified and enriched under specific culture conditions. Second, although the SKPs differentiated into Schwann cells in the microenvironment of the peripheral nerve, only portions of the transplanted cells were induced towards a neural fate, which implies that the pre-differentiation procedure is nec-

essary for the Schwann cell graft application. Third, SKP-SCs can survive and proliferate after being transplanted into the rat sciatic nerve. They were able to robustly integrate into the axonal regeneration and myelination, and induced similar effects on nerve regeneration as primary Schwann cells. Thus, these data suggest that SKP-SCs could be a valuable source for Schwann cell transplantation to resolve the lack of such cells for cell therapy treatments of peripheral nerve injuries.

After peripheral nerve injury, regeneration of the axon can occur spontaneously, but the nerve defect will not likely be recovered without a bridge material. While autologous nerve grafts are considered to be the gold standard for the repair of peripheral nerve defects, the complications associated with this technique limit its clinical application, at least in some patients. A number of nerve conduits have been assessed in the pursuit of a suitable approach for bridging nerve defects. Although reports in the literature have demonstrated that large nerve gaps of various lengths have been regenerated using synthetic or biological conduits. In addition, numerous studies have revealed the potential effects of different cell types for improving the efficacy of nerve conduits transplanted for nerve regeneration. Schwann cells have been shown to enhance axonal regeneration in a rat sciatic nerve injury model. Bone marrow stem cells also likely play an active role in conduit-guided nerve regeneration.

Fernandes et al. (2008) demonstrated that SKPs are progenitor cells with similar molecular and functional properties as embryonic neural crest stem cells. In medium supplemented with serum and growth factors, human SKPs differentiate into both neuronal cells and astrocytes (Toma et al., 2001; Fernandes et al., 2004). Moreover, differentiation culture medium supplemented with growth factors promotes SKPs differentiation towards Schwann cells in significantly higher percentages than other culture systems. Based on these studies, SKP-SCs and SKPs were considered to be an accessible cell source for cell therapy of peripheral nerve injuries. In several studies, SKPs and/or SKP-SCs were seeded into different scaffolds to try to repair nerve gaps after nerve injuries, leading to satisfactory outcomes. However, there are few reports that evaluated the independent function of SKPs and SKP-SCs during nerve regeneration. Herein, we used silicone tubes seeded with different types of seed cells to determine the effects of SKPs, SKP-SCs, and primary Schwann cells.

In this study, we used Schwann cell differentiation medium, DMEM/F12 supplemented with neuregulin-1 and forskolin, to induce the differentiation of SKPs. After 10 days in culture, around 80% of the cells in the Schwann cell differentiation medium expressed S100, which is consistent with the results from prior reports. However, the purity of Schwann cells did not significantly change even after the cultures were passaged several times. When we compared the effects of the different cell types on nerve regeneration, the homogeneity of the cell population is one of the most important factors to arrive at an accurate conclusion. Therefore, differential adhesion was used to increase the

purity of the Schwann cells in culture before cell transplantation. Numerous experiments have shown that differential adhesion can be used to purify and enrich primary Schwann cells in culture without changing their molecular and functional properties. Here, we obtained SKP-SCs at 95% purity in culture using this technique. This result is comparable to the cell purities reported by other studies in which SKP-SCs were assessed as a source for cell transplantation (Belicchi et al., 2004; McKenzie et al., 2006; Marchesi et al., 2007; Walsh et al., 2009).

Previously, SKP-SCs have been shown to proliferate and produce myelin during *in vitro* culture. SKPs or SKP-SCs were transplanted into rats with peripheral nerve injuries or Shiverer mutant mice, and they were integrated into axonal myelination in the peripheral nervous system. Similar results were obtained after cells were transplanted into the central nervous system (McKenzie et al., 2006). In addition, undifferentiated SKPs were seeded into a collagen conduit to bridge nerve defect gaps. Eight weeks after cell transplantation, the immunohistochemistry results showed expression of S100 and myelin basic protein in donor cells, indicating the differentiation of SKPs in peripheral nerve injury conditions, as well as the cell therapy potential of SKPs for nerve regeneration (Marchesi et al., 2007). Recently, nerve-derived Schwann cells or SKP-SCs were seeded into acellular nerve grafts to evaluate the regeneration potential of SKP-SCs after peripheral nerve damage. In their hands, SKP-SCs remarkably enhanced nerve regeneration 4–8 weeks after transplantation when compared with the control group, in which only basal media were used (Walsh et al., 2009). In the present study, 5-mm-long nerve defects were bridged by a silicone tube seeded with nerve-derived SCs, SKPs, or SKP-SCs. Eight weeks after transplantation, the gross findings showed that the nerve gaps were filled in all three groups. However, the diameters of the regenerated nerve within the silicone tube were significantly smaller in the SKP group than in the SKP-SC and SC groups, which had similarly sized regenerated nerves. The number of axons in the regenerated region of the SKP-SC group was similar to that in the SCs group as assessed by immunohistochemistry, whereas the number was greater than in the SKP group. It has been suggested that transplanted SCs enhance nerve regeneration by producing and secreting neurotrophin and cytokines in the host nerve (McKenzie et al., 2006). Here, we found fewer S100-positive cells in the SKP group than in the SC and SKP-SC groups. One major reason for the greater axonal regeneration in the SC and SKP-SC groups may be fewer donor cells with the Schwann cell phenotype found in the SKP group compared with the SKP-SC and SC groups. Moreover, immunostaining showed that there were more GFP-positive cells surrounding the regenerated axons in the SC and SKP-SC groups than in the SKP group. Together with the EMG results, these findings suggest that the percentage of SC-like cells in the donor cell population may be a key factor for successful nerve regeneration in such nerve gap models. In conclusion, SKP-SCs are an accessible alternative for Schwann cells that may be able to improve axonal regeneration after

peripheral nerve injury.

Author contributions: Zhang P obtained the data, conducted the experiments, and performed the data analysis. Lu XC conceived and designed the study and provided technical and material support. Chen ZB wrote the manuscript. Chen JH provided technical support and revised the manuscript. All authors approved the final version of the manuscript.

Conflicts of interest: None declared.

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