

Pre-degenerated peripheral nerves co-cultured with bone marrow-derived cells: a new technique for harvesting high-purity Schwann cells

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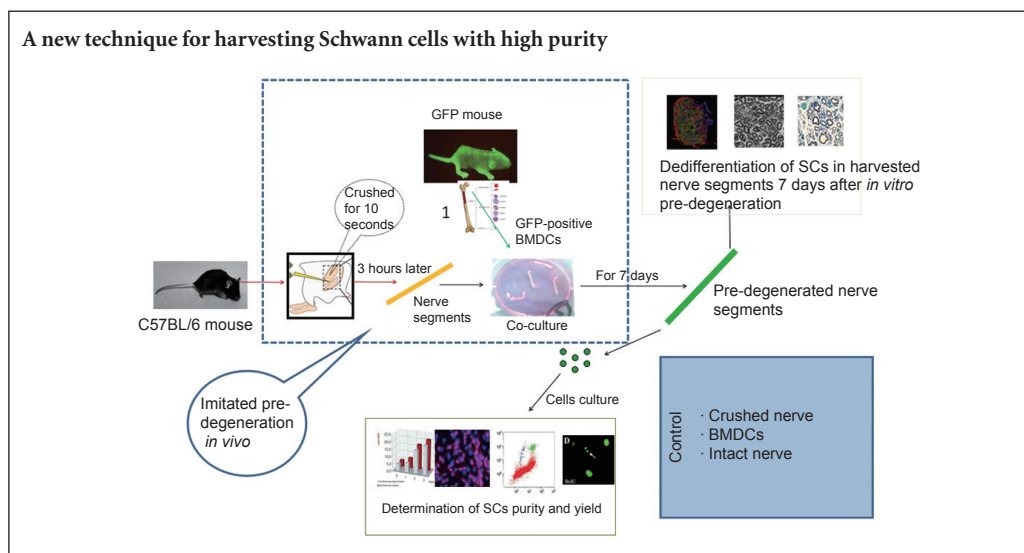
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Graphical Abstract



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Abstract

Schwann cells play an important role in the peripheral nervous system, especially in nerve repair following injury, so artificial nerve regeneration requires an effective technique for obtaining purified Schwann cells. *In vivo* and *in vitro* pre-degeneration of peripheral nerves have been shown to obtain high-purity Schwann cells. We believed that *in vitro* pre-degeneration was simple and controllable, and available for the clinic. Thus, we co-cultured the crushed sciatic nerves with bone marrow-derived cells *in vitro*. Results demonstrated that, 3 hours after injury, a large number of mononuclear cells moved to the crushed nerves and a large number of bone marrow-derived cells infiltrated the nerve segments. These changes promoted the degradation of the nerve segments, and the dedifferentiation and proliferation of Schwann cells. Neural cell adhesion molecule and glial fibrillary acidic protein expression were detected in the crushed nerves. Schwann cell yield was $9.08 \pm 2.01 \times 10^4/\text{mg}$. The purity of primary cultured Schwann cells was $88.4 \pm 5.79\%$. These indicate a successful new method for obtaining Schwann cells of high purity and yield from adult crushed sciatic nerve using bone marrow-derived cells.

Key Words: nerve regeneration; bone marrow-derived cells; Schwann cells; co-culture; *in vitro* pre-degeneration; dedifferentiation; glial fibrillary acidic protein; neural cell adhesion molecule; mononuclear cells; neural regeneration

Introduction

The gold standard for peripheral nerve repair is autologous nerve grafting. However, the disadvantages of this method are that it can result in nerve defects at the donor sites and result in only a limited number of successful nerve grafts. Alternative strategies for peripheral nerve repair include

creating tubular nerve guidance channels from natural or synthetic materials (Mosahebi et al., 2002; Chalfoun et al., 2006; Li et al., 2006; Cho, 2009). However, a key factor for the successful functioning of artificial nerves is the availability of Schwann cells (SCs). To this end, a reliable method for obtaining SCs in culture is needed.

SCs can be obtained from the nerves of neonates as opposed to adult animals, since the former have non-myelinating SCs that can proliferate (Dong et al., 1997; Haastert et al., 2007). Injury to the peripheral nervous system results in Wallerian degeneration of axons, followed by regeneration after macrophages penetrate and remove the damaged myelin sheath (Chen et al., 2015). Subsequently, SCs dedifferentiate and re-enter the cell proliferation cycle, forming processes known as Bungner's bands (Stoll and Muller, 1999) that act as guides for the regenerating axons (Son and Thompson, 1995). Mitosis begins 3–5 days after injury and is accompanied by changes in SCs such as increases in the expression of the neural cell adhesion molecule (NCAM) and the p75 neurotrophin receptor (p75^{NTR}). Cytoskeletal constituents such as glial fibrillary acidic protein (GFAP) and vimentin also increase (Neuberger and Cornbrooks, 1989; Jessen et al., 1990; Martini, 1994). All are essential for nerve regeneration. Wallerian degeneration is a systematic process that involves a variety of cell types. Signals from degenerating axons and SC chemokines and cytokines recruit and activate macrophages from bone marrow (Lee et al., 2006). These produce factors that promote myelin phagocytosis (Boivin et al., 2007) and SC dedifferentiation and proliferation (Muller, 1996; Stoll et al., 2002).

A method for obtaining SC-enriched cultures, based on SC dedifferentiation and proliferation caused by Wallerian degeneration, has been described (Stoll and Muller, 1999). The distal stump of a nerve was left in the animal for several days before being harvested for implantation or culture, by a process known as *in vivo* pre-degeneration. After dissociation and culture, SCs of highly purity and proliferative capability were obtained (Keilhoff et al., 1999; Komiyama et al., 2003; Mauritz et al., 2004; Pannunzio et al., 2005). However, *in vitro* pre-degeneration involves the dissection, dissociation, and culture of nerve segments for 7 or more days (Kraus et al., 2010). Although the efficiency is low for *in vitro* degeneration compared with *in vivo*, it is simple and controllable, therefore more suitable for clinical application. To increase the efficiency of *in vitro* methods, the present study investigated whether bone marrow-derived cells (BMDCs) can promote *in vitro* pre-degeneration of peripheral nerves and subsequent activation of SCs. We established a new model in which the sciatic nerve of mice was crushed for 3 hours, leading to the recruitment of mononuclear cells. Crushed nerve segments were then co-cultured with BMDCs to induce *in vitro* pre-degeneration. We then evaluated the degree of degeneration, dedifferentiation and proliferation of SCs as well as their purity and yield.

Materials and Methods

Animals

Sixty-four healthy male and female, adult (7–8 weeks) C57BL/6 mice, weighing 23–27 g, and six male and female adult (7–8 weeks) C57BL/6-Tg (CAG-EGFP) C14-Y01-FM1310sb mice expressing green fluorescent protein (GFP), weighing 22–25 g, were provided by Shanghai SLAC Laboratory Animal Co., Ltd., China (license No. SYXK (Hu)

2012-0001). All mice were housed in the Central Laboratory of Bengbu Medical University of China in a 12-hour light/dark cycle at 22°C, with a humidity of 40–67%, under specific-pathogen-free conditions. All procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China (2006). Animal experiments were approved by the Institutional Review Committee of Bengbu Medical College of China.

Isolation of mouse BMDCs

BMDCs were obtained from femur and tibia bone marrow of the six transgenic GFP mice. The bone marrow was lysed by adding erythrocyte lysis buffer and extracted in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Hyclone) and 1% penicillin/streptomycin. Homogeneous cell suspensions were then reserved for co-culture with sciatic nerve segments.

Sciatic nerve segments

Sciatic nerve segments were harvested from 64 7–8-week-old C57BL/6 mice that were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg). The sciatic nerves were exposed bilaterally and one side crushed from the distal to the proximal end across the sciatic notch for 10 seconds with fine forceps. Three hours later, a nerve segment was grasped with micro tweezers and proximally dissected up to its point of transition into the lumbar plexus where it was cut. Distally directed dissection of the nerve was carried out beyond the level of the knee to the end of the skin incision, where a distal cut was made. The contralateral uncrushed sciatic nerve was harvested as a control.

In vitro pre-degeneration

After removal of connective tissue, the nerve segments were placed in a 6-well plate with pre-degeneration medium consisting of DMEM, 10% FBS, and 1% penicillin/streptomycin. The medium was changed every 2 days throughout the 7-day pre-degeneration process. Samples were assigned to four groups ($n = 8$ nerve segments each). In the crushed nerve + BMDCs group, the BMDCs suspensions were first seeded in a 6-well plate, and then crushed nerves were placed directly for co-culturing with BMDCs. In the crushed group, crushed nerves were cultured in medium only. In the BMDCs group, intact nerves were co-cultured with BMDCs. In the intact nerve group, intact nerves were cultured in medium only.

Immunohistochemistry

Following pre-degeneration, part of each sciatic nerve was rinsed with 0.1 M phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde. The tissue was cryoprotected by immersion in increasing concentrations of sucrose (10–30%) over several days, then blocked, embedded in optimal cutting temperature compound (SAKURA, Oakland, CA, USA), and frozen on dry ice. Frozen specimens were sectioned on a cryostat at a thickness of 12 μ m in the longitudinal plane,

and serial sections were collected on Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA, USA). The sections were washed in PBS, permeabilized with 0.3% Triton X-100, blocked with 10% goat serum in PBS, and incubated overnight with the following primary antibodies against dedifferentiated SCs: p75^{NTR}, NCAM, and GFAP (all rabbits, 1:1,000; Dako, Glostrup, Denmark), as well as antibodies against macrophage/mononuclear cells, *i.e.*, F4/80 and SMI-94 (both 1:200; Sternberger Monoclonals Inc., Baltimore, MD, USA). Alexa Fluor 546 secondary antibodies (goat and rabbit 1:1,000; Invitrogen, Carlsbad, CA, USA) were used to visualize immunoreactivity, and incubated for 30 minutes at 37°C. Samples were observed and photographically imaged with a confocal laser scanning microscope (TCS SP5, Leica, Germany). The fluorescence intensities of p75^{NTR}, NCAM, and GFAP were analyzed with Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA) in each group from at least three random photo-areas at 200× magnification for each experiment. GFP-positive cells were also counted.

Transmission electron microscopy

Two nerve segments for electron microscopy were obtained at the end of pre-degeneration in each group. Tissue specimens were fixed in 1% buffered glutaraldehyde/1% paraformaldehyde and processed as previously described. Ultra-thin sections were cut and analyzed with a transmission electron microscope (JEOL, Tokyo, Japan). The intact myelin was counted in each group from at least three random photo-areas at 1,000× magnification for each experiment.

Cell culture

An enzyme solution for tissue digestion was prepared by dissolving collagenase NB4 (Serva, Heidelberg, Germany) in DMEM at a concentration of 0.2% (0.27 U/mL). After 7 days of pre-degeneration, sciatic nerve samples were rinsed with PBS and cut into 2-mm pieces. After adding enzyme solution, samples were incubated at 37°C for 60 minutes. An

equal volume of 0.05% trypsin-EDTA (Gibco, Grand Island, NY, USA) was added to the reaction. The nerve segments were mechanically dissociated by pipetting for 3–5 minutes. A 10-mL volume of FBS was added to stop the digestion and the mixture was centrifuged at 600 × *g* for 10 minutes. After removing the supernatant, the cell pellet was resuspended in SC culture medium consisting of DMEM supplemented with 10% FBS, 2 μM forskolin (Sigma, St. Louis, MO, USA), 10 ng/mL heregulin-β-1 (PeproTech, Rocky Hill, NJ, USA), and 50 ng/mL basic fibroblast growth factor (PeproTech). The cell suspension was seeded in a laminin-coated 25-cm² flask at a density of 2.0 × 10⁴ cells/cm² and incubated at 37°C in a humidified atmosphere of 5% CO₂.

Morphological observation of SCs

SC morphology was assessed by phase contrast microscopy (Olympus, Tokyo, Japan) at 100× magnification. Cells obtained by enzymatic digestion were resuspended in SC culture medium. Most cells adhered to the laminin-coated flasks within 48 hours and had one of two distinct shapes corresponding to two cell types: SCs were small, bipolar or tripolar, and refractile, while fibroblasts had a flat, polygonal shape with an oval nucleus and blunt cytoplasmic processes.

Immunocytochemistry

Cells cultured on cover slips were fixed with 4% paraformaldehyde for 20 minutes, washed three times with PBS, and then blocked with 10% goat serum (Sigma) in PBS for 30 minutes at 37°C. Samples were treated with rabbit polyclonal anti-p75^{NTR} antibody (Abcam, Cambridge, UK) diluted 1:500 in PBS at 37°C for 1 hour. As a control, samples were incubated without primary antibody. After three washes with PBS, samples were treated with Alexa Fluor 546 goat anti-rabbit IgG (Invitrogen) diluted 1:1,000 in PBS for 30 minutes at 37°C. Cell nuclei were counterstained with 1 μM Hoechst 33343 (Sigma) for 10 seconds. After a final wash in

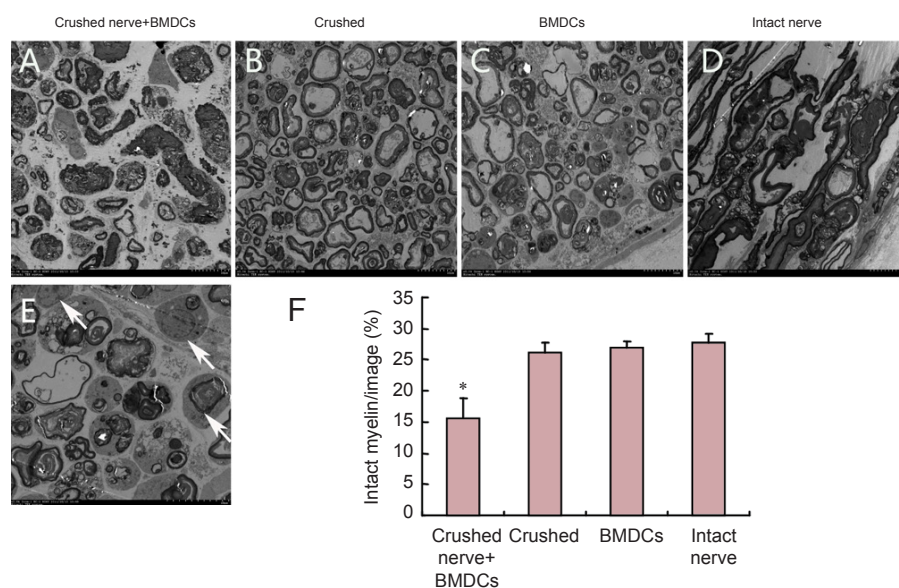


Figure 3 Ultrastructural changes in nerves 7 days after *in vitro* pre-degeneration (transmission electron microscopy).

(A–D) Axons and myelin sheaths were degenerated to a greater extent in the crushed nerve + BMDCs group (A) as compared with the crushed, BMDCs and intact nerve groups (B–D). (E) Phagocytic cells (white arrows) were also observed in the crushed nerve + BMDCs group, but not in the crushed, BMDCs and intact nerve groups. Scale bars: 10 μm. (F) Quantitative analysis of intact myelin in nerve segments. **P* < 0.05, vs. crushed, BMDCs and intact nerve groups (mean ± SD, one-way analysis of variance and the least significant difference test). Crushed nerve + BMDCs group: Crushed nerve co-cultured with BMDCs; crushed group: crushed nerve cultured in medium only; BMDCs group: intact nerve co-cultured with BMDCs; intact nerve group: intact nerve cultured in medium only; BMDCs: bone marrow-derived cells.

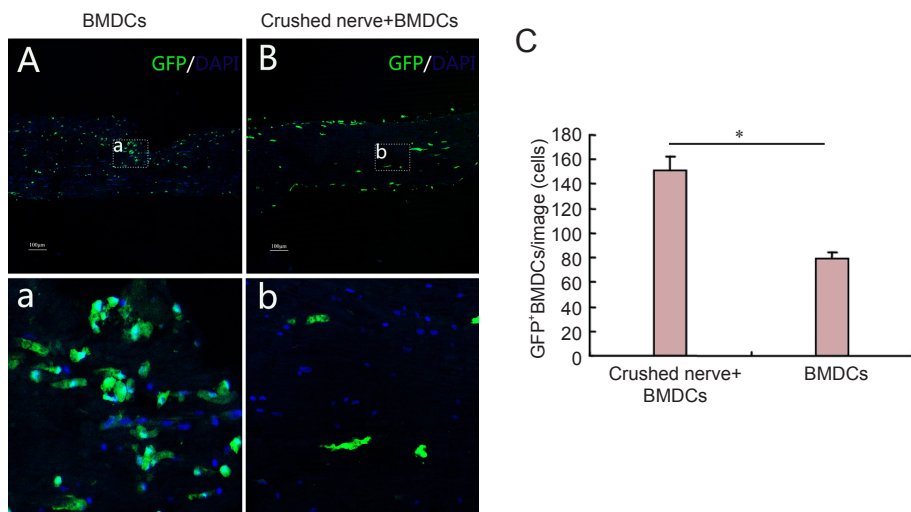


Figure 2 BMDCs infiltration into harvested nerve segments after 7 days of *in vitro* pre-degeneration (confocal laser scanning microscope).

(A, B) A large number of GFP-positive BMDCs were observed in the harvested nerve segment in crushed nerve + BMDCs group (A) and BMDCs group (intact nerve co-cultured with BMDCs) (B). Scale bars: 100 μ m. (a, b) Magnified views of areas enclosed by broken lines in panels A and B, respectively. (C) Quantitative analysis of the number of GFP-positive cells. * $P < 0.05$ (mean \pm SD, two-sample *t*-test). GFP: Green fluorescent protein; DAPI: 4',6-diamidino-2-phenylindole; BMDCs: bone marrow-derived cells.

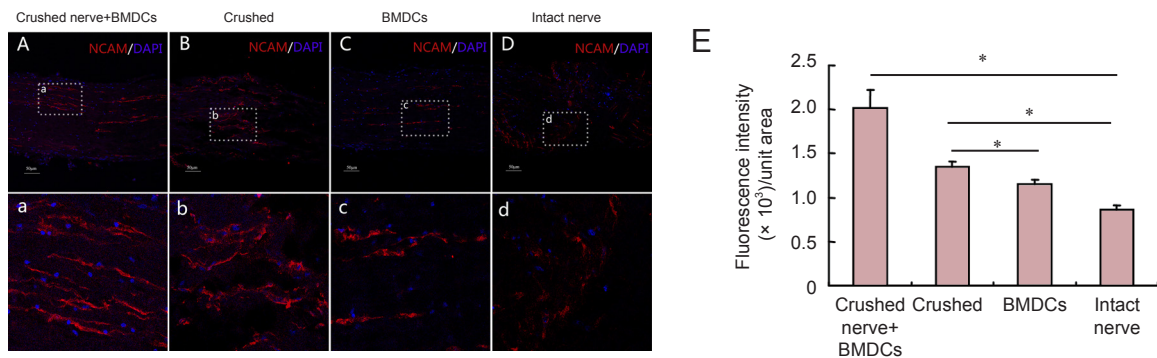


Figure 4 Dedifferentiation of SCs in harvested nerve segments 7 days after *in vitro* pre-degeneration (immunofluorescence staining, confocal laser scanning microscope).

(A–D) Dedifferentiated SCs positive for NCAM expression and stained with DAPI. Scale bars: 50 μ m. (a–d) Magnified views of areas enclosed by broken lines in panels A–D. (E) Quantitative analysis of NCAM expression (fluorescence intensity). * $P < 0.05$ (mean \pm SD, one-way analysis of variance and least significant difference test). Crushed nerve + BMDCs group: Crushed nerve co-cultured with BMDCs; crushed group: crushed nerve cultured in medium only; BMDCs group: intact nerve co-cultured with BMDCs; intact nerve group: intact nerve cultured in medium only; NCAM: neural cell adhesion molecule; DAPI: 4',6-diamidino-2-phenylindole; SCs: Schwann cells; BMDCs: bone marrow-derived cells.

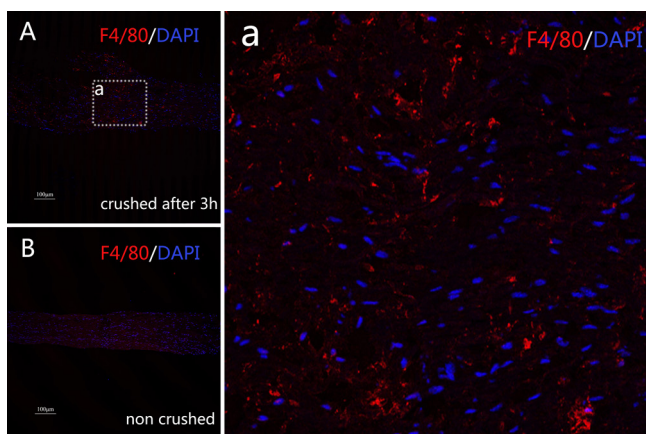


Figure 1 Mononuclear cells in harvested nerve segments 3 hours after injury (immunofluorescence staining, confocal laser scanning microscope).

(A) F4/80 and DAPI staining shows F4/80-immunoreactive mononuclear cells recruited to the nerve 3 hours after crush injury. Scale bars: 100 μ m. (B) Few F4/80-immunoreactive cells were observed in intact nerves. (a) Magnified view of the area enclosed by broken lines in panel A. F4/80: Mouse epidermal growth factor-like module-containing mucin-like hormone receptor-like 1; DAPI: 4',6-diamidino-2-phenylindole.

PBS, samples were mounted with mounting medium (Dako) and visualized under a fluorescence microscope (Olympus). Images were captured and processed with Image-Pro Plus software (Media Cybernetics). The cell morphologies were observed and classified, and the SC purity was calculated based on p75^{NTR} immunostaining.

Determination of SC purity and yield

After immunostaining with antibodies, SCs and fibroblasts were identified based on their morphologies. Cells with a bipolar or tripolar shape were recognized as SCs, whereas flat or polygonal cells were recognized as fibroblasts. The purity of SCs was derived from the calculation of the percentage of p75^{NTR}-positive cells with respect to the total number of counted Hoechst-positive cells:

(1) SCs purity (%) = p75^{NTR}-positive cells/Hoechst-positive cells \times 100%.

(2) Cell yield was determined by cell counts using a hemocytometer following trypsinization and then the final cell yield was calculated as follow: Cell yield (10^4 /mg) = number of cells/weight of nerve segments in each group.

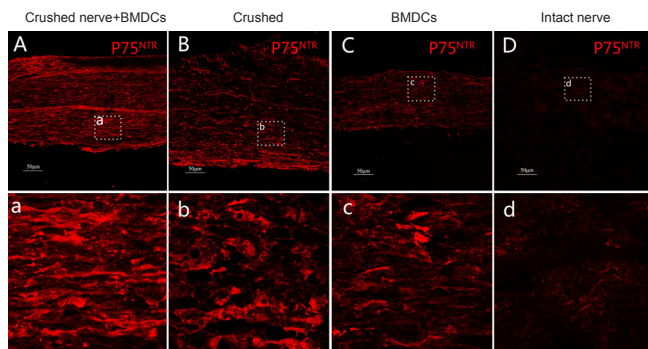


Figure 5 Dedifferentiation of SCs in harvested nerve segments 7 days after *in vitro* pre-degeneration (immunofluorescence staining, confocal laser scanning microscopy).

(A–D) Dedifferentiated SCs positive for p75^{NTR} expression and stained with DAPI. Scale bars: 50 μ m. (a–d) Magnified views of areas enclosed by broken lines in panels A–D. (E) Quantitative analysis of p75^{NTR} expression (fluorescence intensity). * $P < 0.05$ (mean \pm SD, one-way analysis of variance and least significant difference test). Crushed nerve + BMDCs group: Crushed nerve co-cultured with BMDCs; crushed group: crushed nerve cultured in medium only; BMDCs group: intact nerve co-cultured with BMDCs; intact nerve group: intact nerve cultured in medium only; p75^{NTR}: p75 neurotrophin receptor; DAPI: 4',6-diamidino-2-phenylindole; SCs: Schwann cells; BMDCs: bone marrow-derived cells.

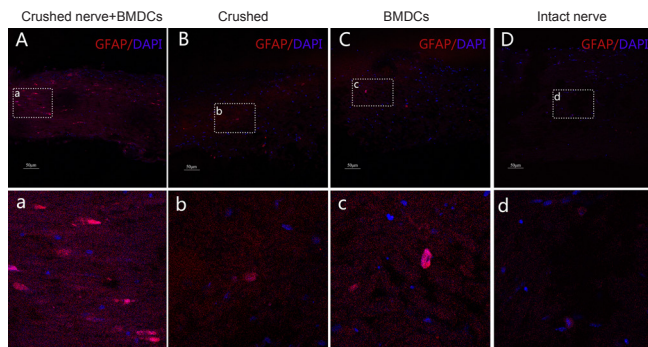
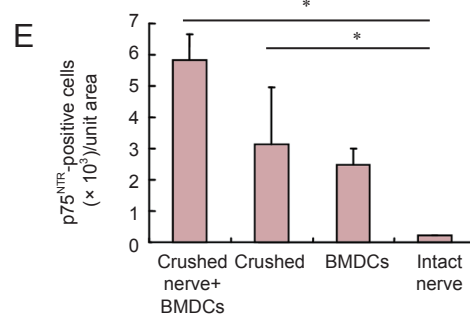


Figure 6 Dedifferentiation of SCs in harvested nerve segments 7 days after *in vitro* pre-degeneration (immunofluorescence staining, confocal laser scanning microscopy).

(A–D) Dedifferentiated SCs positive for GFAP expression and stained with DAPI. Scale bars: 50 μ m. (a–d) Magnified views of areas enclosed by broken lines in panels A–D. Crushed nerve + BMDCs group: Crushed nerve co-cultured with BMDCs; crushed group: crushed nerve cultured in medium only; BMDCs group: intact nerve co-cultured with BMDCs; intact nerve group: intact nerve cultured in medium only; GFAP: glial fibrillary acidic protein; DAPI: 4',6-diamidino-2-phenylindole; SCs: Schwann cells; BMDCs: bone marrow-derived cells.

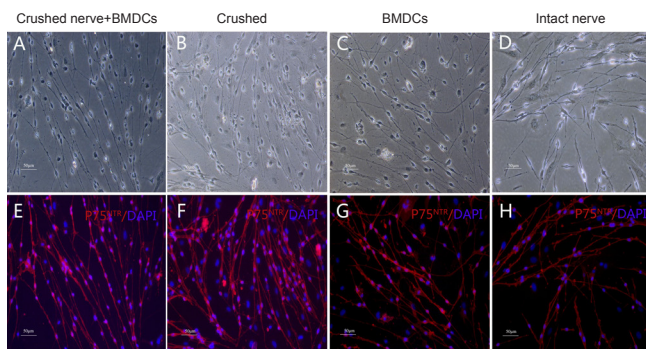


Figure 7 SCs after primary culture for 48 hours.

(A–H) SCs showed bipolar or tripolar morphology by phase contrast microscopy (A–D) and were positive for p75^{NTR} expression under a fluorescence microscope (E–H), with a high degree of co-localization (D). Meanwhile, fibroblasts were flat and polygonal with an oval nucleus and blunt cytoplasmic processes (A–D) and were negative for p75^{NTR} (E–H). Most cells in the crushed nerve + BMDCs group (A, E) were SCs. Crushed nerve + BMDCs group: Crushed nerve co-cultured with BMDCs; crushed group: crushed nerve cultured in medium only; BMDCs group: intact nerve co-cultured with BMDCs; intact nerve group: intact nerve cultured in medium only; p75^{NTR}: p75 neurotrophin receptor; SC: Schwann cells; BMDCs: bone marrow-derived cells. Scale bars: 50 μ m.

Statistical analysis

All data are expressed as the mean \pm SD. For quantitative comparison and analysis, the values were subjected to two-sample *t*-test, one-way analysis of variance and the least significant difference test using SPSS 18.0 software (SPSS, Chicago, IL, USA). Statistical significance was set at $P < 0.05$.

Results

Mononuclear cells were recruited to the site of nerve injury

Following injury to the peripheral nerve, SC- and macrophage-derived cytokines and chemokines recruit mononuclear cells from the blood that phagocytose debris while activating SCs during Wallerian degeneration. Indeed, we observed many F4/80-positive cells in crushed nerves, but

not in intact nerves (Figure 1).

BMDCs infiltrated into nerve segments in co-cultures with injured nerve segments

BMDCs were obtained from transgenic GFP mice so that they could be visualized in co-cultures with harvested nerve segments. We observed many infiltrated GFP-positive cells in nerve segments of crushed nerve + BMDCs and BMDCs groups, particularly in the crushed nerve + BMDCs group ($P < 0.05$; Figure 2).

BMDCs promoted the degeneration of co-cultured injured nerve segments

Nerve segments in all groups showed degeneration 7 days

Table 1 Cell yield in the four experimental groups 7 days following *in vitro* pre-degeneration and purity of SCs after primary culture for 48 hours

Group	Cell yield (10^4 /mg)	SCs purity (%)
Crushed nerve + BMDCs	9.08±2.01*	88.4±5.79*
Crushed	5.72±0.56	74.2±4.06
BMDCs	5.86±1.94	79.2±2.65
Intact nerve	4.96±1.83	76.1±3.72

* $P < 0.05$, vs. crushed, BMDCs and intact nerve groups (mean ± SD, one-way analysis of variance and least significant difference test). Crushed nerve + BMDCs group: Crushed nerve co-cultured with BMDCs; crushed group: crushed nerve cultured in medium only; BMDCs group: intact nerve co-cultured with BMDCs; intact nerve group: intact nerve cultured in medium only; SCs: Schwann cells; BMDCs: bone marrow-derived cells.

after *in vitro* pre-degeneration, including of axons and myelin sheaths. The extent of degeneration and the number of phagocytic cells were greater in the crushed nerve + BMDCs group than in the crushed nerve only, BMDCs and intact nerve groups ($P < 0.05$; **Figure 3**). There were no differences in the degree of degeneration or the number of phagocytic cells among the crushed, BMDCs and intact nerve groups.

BMDCs promoted the dedifferentiation of SCs from injured nerve segments

During Wallerian degeneration, SCs re-expressed embryonic cell surface molecules, such as NCAM and p75^{NTR}, and upregulated cytoskeletal components, such as GFAP as a part of the dedifferentiation process. We detected NCAM, p75^{NTR}, and GFAP expression in SCs in all groups 7 days after pre-degeneration, with the highest levels observed in crushed nerve + BMDCs group (**Figures 4–6**). The levels of NCAM, p75^{NTR}, and GFAP proteins were similar among crushed, BMDCs and intact nerve groups.

Characterization of dedifferentiated SCs

Nerve segments were dissociated 7 days after *in vitro* pre-degeneration and the number of cells was counted before seeding in 6-well plates. The cumulative cell counts were significantly higher in the crushed nerve + BMDCs group than in the crushed, BMDCs and intact nerve groups ($P < 0.05$; **Table 1**).

After enzymatic dissociation, cells were resuspended in SC culture medium; most adhered to flasks within 48 hours and had one of two shapes. Fibroblasts were flat and polygonal with an oval nucleus and blunt cytoplasmic processes, while SCs were small, elongated, fusiform, and bipolar or tripolar. Based on these characteristics, most cells in the crushed nerve + BMDCs group were SCs and few were fibroblasts. The percentage of fibroblasts was higher in the crushed, BMDCs and intact nerve groups than in the crushed nerve + BMDCs group. The identity of SCs was confirmed by epifluorescence microscopy using antibodies against p75^{NTR} (**Figure 7**). Primary cultures of SCs purity was significantly higher in the crushed nerve + BMDCs group (88.4% ± 5.79% based on P75^{NTR} expression) than that in the crushed, BMDCs and

intact nerve groups ($P < 0.05$; **Table 1**).

Discussion

In the present study, we designed a new model for *in vitro* pre-degeneration. *in vivo* pre-degeneration is an efficient method for culturing SCs (Keilhoff et al., 1999; Tomita et al., 2009). However, *in vivo* pre-degeneration requires two separate surgical procedures and is unacceptable in humans for ethical reasons. Thus, the *in vitro* approach may be widely applicable, because it is simple and controllable, although the efficiency is low. Therefore, we designed our new model of crushed nerve segments co-cultured with BMDCs so as to imitate the *in vivo* pre-degeneration.

In this study, the new model imitated the partial process of *in vivo* pre-degeneration successfully, in which mononuclear cells are recruited to the crushed nerve. Moreover, BMDCs infiltrated nerve segments 7 days after co-culture, which was consistent with the process of *in vivo* pre-degeneration. It was previously shown that peripheral nerves crushed for 3 hours and co-cultured with BMDCs induced the expression of leukemia inhibitory factor and interleukin-6 in SCs. This was followed by activation and recruitment of blood mononuclear cells, which may in turn recruit BMDCs and promote SC dedifferentiation and proliferation (Tofaris et al., 2002). Matrix metalloproteinases have been shown to play a critical role in this process (La Fleur et al., 1996). In particular, matrix metalloproteinase-9 expression after a nerve is crushed is associated with the breakdown of the blood-nerve barrier and mononuclear cell invasion (Shubayev and Myers, 2000; Siebert et al., 2001). Taking these points into consideration was crucial for our study to succeed.

During nerve degeneration, cytokines released by mononuclear phagocytes are critical for SC dedifferentiation and re-entry to the cell cycle for their proliferation; all necessary for the culture of SCs. For example, interleukin-1 stimulates nerve growth factor production (Lindholm et al., 1987, 1988), and induces the expression of integrin- β 1 on fibroblasts-like cells (Santala and Heino, 1991). Integrin- β 1 is temporally correlated with macrophage recruitment to the endoneurium (Taskinen et al., 1995). Transforming growth factor- β , another macrophage-derived cytokine, is a mitogen for SCs (Ridley et al., 1989). Moreover, transforming growth factor- β released by SCs acts a macrophage chemoattractant (Stoll et al., 1989). Thus, mutually regulatory interactions exist between macrophages and SCs.

In our model, mononuclear cells were recruited and BMDCs were infiltrated in the crushed nerve segments and clearly promoted the degeneration of the nerve segments. The axons and the myelin sheets were disrupted and then removed. SCs dedifferentiated and proliferated, regaining the expression of NCAM and p75^{NTR} and upregulating GFAP. This cell culture has proved an effective technique to obtain Schwann cells. In our study, the cumulative cell count in the culture was $9.08 \pm 2.01 \times 10^4$ /mg and the purity was 88.4 ± 5.79 % in primary culture.

In summary, our study demonstrates a method for obtain-

ing a highly pure SC culture from crushed sciatic nerve by co-culturing with BMDCs that can potentially be used for artificial nerve regeneration following nerve injury.

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Author contributions: XPW designed the study, conducted the experiments, analyzed the data, and wrote the paper. MW obtained the funding and provided the critical revision of the paper. JZG designed the study. ZDW and XBG participated in nerve pre-degeneration and co-culture with BMDCs. YYL participated in cell culture and animal administration. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Plagiarism check: This paper was screened twice using CrossCheck to verify originality before publication.

Peer review: This paper was double-blinded and stringently reviewed by international expert reviewers.

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