Study on the adoption of Schwann Cell Phenotype by Bone Marrow Stromal Cells *in vitro* and *in vivo*[1]

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Objective To explore the possibilities of bone marrow stromal cells (MSCs) to adopt Schwann cell phenotype *in vitro* and *in vivo* in SD rats. **Methods** MSCs were obtained from tibia and femur bone marrow and cultured in culture flasks. Beta-mercaptoethanol followed by retinoic acid, forskolin, basic-FGF, PDGF and heregulin were added to induce differentiation of MSCs'. Schwann cell markers, p75, S-100 and GFAP were used to discriminate induced properties of MSCs' by immunofluorescent staining. PKH-67-labelled MSCs were transplanted into the mechanically injured rat sciatic nerve, and laser confocal microscopy was performed to localize the PKH67 labelled MSCs in the injured sciatic nerve two weeks after the operation. Fluorescence PKH67 attenuation rule was evaluated by flow cytometry *in vitro*. **Results** MSCs changed morphologically into cells resembling primary cultured Schwann cells after their induction *in vitro*. *In vivo*, a large number of MSCs were cumulated within the layer of epineurium around the injured nerve and expressed Schwann cell markers, p75, S-100, and GFAP. **Conclusion** MSCs are able to support nerve fiber regeneration and re-myelination by taking on Schwann cell function, and can be potentially used as possible substitutable cells for artificial nerve conduits to promote nerve regeneration.

Key words: Bone marrow stromal cells; Differentiation; Schwann cells

INTRODUCTION

Bone marrow stromal cells (MSCs) are well known multi-potential stem cells, which can differentiate into several cell types such as osteoblasts, adipocytes, chondrocytes, and even muscle cells under specific condition. Although MSCs are quite different from neuronal lineage, their potential to develop into neurons and astrocytes both *in vivo*[1] and *in vitro*[2-3] has been reported. MSCs have the capacity of differentiating into myelin-forming cells *in vivo* and repairing demyelinated spinal cord axons[4]. Mari *et al.*[5] and Pedro *et al.*[6] have verified its abilities to facilitate nerve regeneration by injecting MSCs directly into the nerve injury site. These findings suggest that MSCs can differentiate into nerve cells under appropriate *in vivo* conditions. Based on these research findings, we cultured and enriched MSCs to the extent of about 10^7 *in vitro* and injected them into mechanically injured peripheral nerves. Our study demonstrates that MSCs can be induced into Schwann cells *in vitro*, and can survive and adopt Schwann cell characteristics of peripheral nerve *in vivo*, expressing GFAP, S-100, and P75.

MATERIALS AND METHODS

Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Chinese Academy of Sciences.

Isolation of MSCs

MSCs were isolated from adult SD rats as previously described[5-6]. The tibia and femur bone marrow was extruded with α-MEM (Gibco) after the rats were sacrificed by overdose pentobarbital (40 mg/kg). The exudation fluid was cultured in α-MEM supplemented with 10% fetal bovine serum (Gibco) and 200 mg/mL Kanamycin at 37℃, 95% humidity in the atmosphere containing 5% CO_2. 10 ng/mL leukemia inhibitory factor (LIF, Sigma, US) was

added to maintain the differentiation potential of MSCs[7]. To get rid of the hematic cells, the culture medium was replaced twice after 48 hours. The adhered cells were cultured and passaged.

Induction of MSCs

Subcultured MSCs were incubated in α-MEM medium supplemented with 1 mmol/L β-mercaptoethanol (β-ME，Sigma) for 24 hours. After the medium was removed and washed with PBS, MSCs were cultured with 10% fetal bovine serum and 35 ng/mL all-trans-retinoic acid (RA, Sigma) for 3 days. Subsequently MSCs were incubated in α-MEM supplemented with 10% fetal bovine serum and compound containing 5 mmol/L forskolin (FSK, Sigma), 10 ng/mL recombinant human basic-fibroblast growth factor (bFGF, Peprotech, London, UK), 5 ng/mL recombinant human platelet derived growth facor-AA (PDGF-AA, Peprotech, London, UK), and 200 ng/mL recombinant human heregulin-β (HRG-β, R&D System, Minneapolis, MN) for 7 days without any passage.

Immunocytochemistry

Induced MSCs were washed in PBS and fixed with 4% formaldehyde in PBS at room temperature for 20 minutes. Immunocytochemistry was performed to discriminate the differentiation characteristics using the following primary antibodies against S-100, p75, and GFAP (rabbit anti rat, Sigma). Primary antibodies were detected with rhodamine (TRITC)-conjugated, affinipure labelled, goat anti-rabbit IgG (H+L) antibody (Jackson Immunoresearch, US). A double-stranded DNA specific fluorescent dye, 4', 6'-diamidino-2-phenylindole hydrochloride (DAPI), was used for cell nuclei counterstaining.

Animal Preparation and Transplantation

The adhered cells were cultured and passaged to about 6×10^7 in α-MEM supplemented with 10% fetal bovine serum and 10 g/mL LIF. MSCs were collected and labelled with PKH67, a well-known fluorescent probe for *in vitro* and *in vivo* cell tracking, following the protocol provided by the manufacturer (Sigma, US). The labeled cells were then divided into two bottles: one for transplantation, the other for green fluorescence attenuation assessment flow cytometry (Beckman) under the same culture conditions described above.

SD rats (about 200g, *n*=6) were anesthetized with pentobarbital sodium (40 mg/kg body weight). A mechanical force was applied to sciatic nerve (chose right side) and nerve fibers were disrupted while epineurium remained with hemostat pincers[21].

MSCs (0.1 mL in α-MEM, about $\times 10^7$ cells /inject site) were injected into the injured site, and the exact injured site was wrapped with an artificial absorbable chitin nerve conduit (inner diameter 1.5 mm and 10 mm long, Patented, National Patent Administration, China, 2002, Number: 01136314.2) to obtain a relative fitting differentiation microenvironment. As control, 0.1 mL of normal saline solution was injected into the left side.

Immunofluorescence Staining

The animals were sacrificed two weeks after injection of MSCs. The injured nerves were embedded in OCT (frozen section-embedding agent), and taken to the cryostat for continuous sectioning, then fixed with 4% paraformaldehyde immediately.

The fixed sections were stained with rabbit Abs (anti-rat-GFAP, anti-rat-P75 and anti-rat-S-100), then with TRITC-conjugated affinipure goat anti-rabbit Ab IgG (H+L). All stained specimens were observed under Olympus XI 70, and a Leica laser confocal microscopy was performed after the specimen were restained with DAPI.

RESULTS

*Morphological Characteristics of Cultured MSCs Before and After Induction*

The morphological characteristics of un-induced MSCs were considerably disarrayed, and loosely attached. The morphology of induced MSCs was much more regular than that of un-induced MSCs under phase-contrast microscope (Fig. 1).

Immunofluorescence Staining After Induction of MSCs

Immunofluorescence staining was carried out to evaluate the nature of induced MSCs and un-induced MSCs using P75, GFAP and S-100, all being known as markers of Schwann cells. Our results showed that only induced MSCs were positive for P75, GFAP and S-100 (Fig. 2)

Flow Cytometry Analysis of PKH67 Labelled Green MSCs in vitro

PKH dyes are probes for *in vitro* and *in vivo* cell tracking. It has been reported that PKH dyes bind irreversibly to cell membranes without significantly affecting cell growth in many cell types. An important feature is that upon cell division, the probe is partitioned equally between each daughter cell, making it possible to quantify cell fluorescence by flow cytometry. In this study, we used flow cytometry
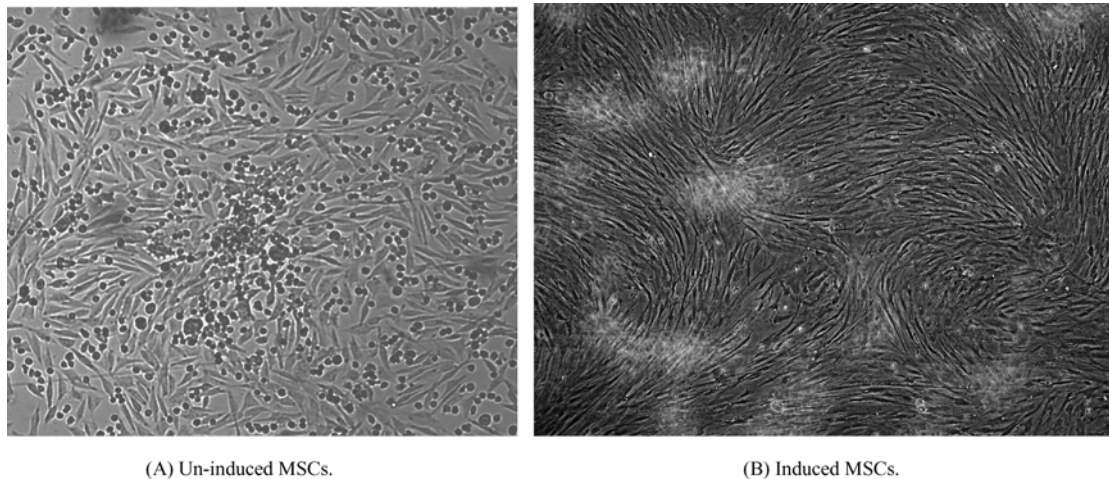
(A) Un-induced MSCs.   (B) Induced MSCs.

FIG. 1. Morphological difference of un-induced MSCs (A) and induced MSCs (B) under phase-contrast microscope.

analysis to measure the fluorescence density of PKH67-labelled MSCs *in vitro*. Data at the end of three-week analysis, depicted in Fig. 3 revealed that the green fluorescence of PKH67 labelled MSCs attenuated gradually during the 3-week culture. The decrease started from 92.16% of MSCs labelled with PKH67 immediately after transplantation, to 82.74% after one week, 75.92% after two weeks later, and 35.26% after three weeks. The result implied that there was still plenty green fluorescence (72.92%) remaining for detection at the transplantation site when we sacrificed the animals two weeks after operation.

Distribution of Grafted Cells

The patterns of distribution of the grafted cells were similar in all six experiments. Grafted cells were observed mainly at the site of injury, where the nerve fiber structure was destroyed. It is very interesting that we also observed that there was green fluorescence penetrated into the adjacent area of the injured site, where the nerve fiber retained the normal structure. The grafted cells, which expressed green fluorescence, were distributed around the injured nerve sites. Fig. 4 shows the proximal nerve segment of damaged nerve site adjacent to the normal nerve site.

Immunofluorescence Staining on Sections of Transplantation

The cell-type markers used to confirm Schwann cell differentiation were GFAP, S-100, and P75, which were regarded as the marker of peripheral nerve glial cells, namely Schwann cells. The injected cells labelled with the PKH67, emitted green fluorescence, while expressions of GFAP, S-100, and

P75 were detected with red fluorescence. Therefore, the incorporated MSCs-derived cells expressing GFAP, S-100, and P75, should express yellow fluorescence (indicated by white arrows in Fig. 5), while the injected cells not expressing the glial-specific markers expressed only green fluorescence after immunofluorescence staining. These data demonstrated that incorporated MSCs adopted the phenotype of Schwann cells. Representative data from six independent experiments are shown in Fig. 5.

DISCUSSION

Schwann cells contribute to nerve regeneration by providing regeneration strands, several kinds of nerve growth factors and tight junctions to stabilize cell contact with injured nerve cells, as well as gap junctions to facilitate traffic of substances between the cells[8]. Although the function of Schwann cells in facilitating nerve regeneration has been confirmed, the limited Schwann cell resource and the xenogenic graft immunorejection reaction hinder its further application. It has been reported that MSCs can differentiate into various lineage cells[9-13]. Junko Hori[14] reported that neural progenitor cells lack immunogenicity and can survive in non-immune-privileged sites. Yuehua Jiang[7] reported that MHC-II is negative in MSCs. Shengkun Sun[15] reported that MSCs fail to express MHC-II and T-cell co-stimulatory molecules CD80 and CD86. Minoru Tomita[16] reported that PKH67-labelled bone marrow-derived stem cells can differentiate into retinal cells in the injured rat retina. Considering all these facts, we observed the biological characteristics of MSCs in non-immune-privileged sites and examined
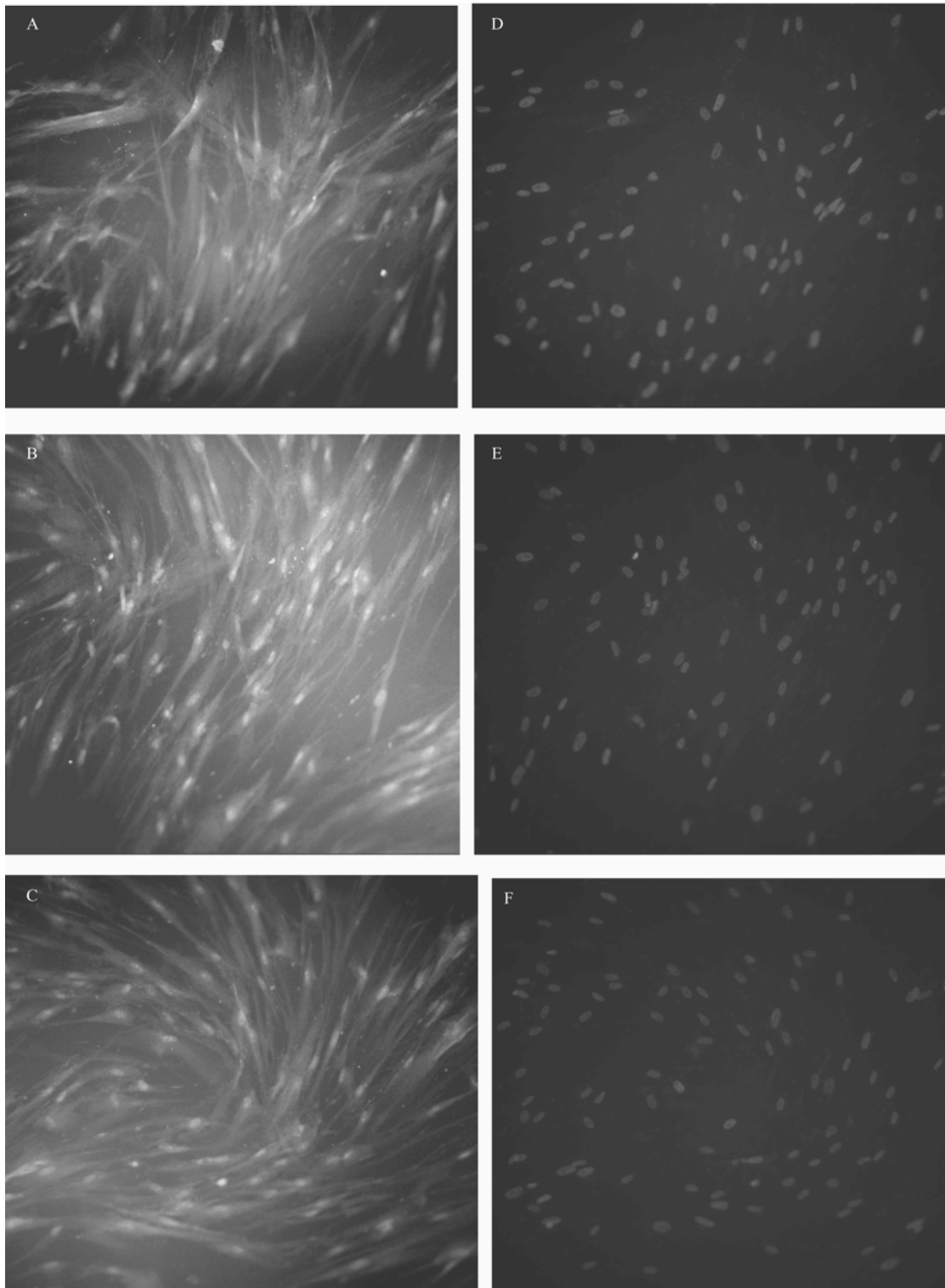
FIG. 2. Immunohistochemical localization of S-100 (A), GFAP (B), and p75 (C) in differentiated MSCs, and corresponding nuclei counterstained with DAPI (D, E, and F). (100×)

A.

PKH67.001

Side soccater / *Forward Scatter*

PKH67.001

Counts / FITC

File: PKH67.001
Gate: No Gate

Marker	Events%	Gated%	Total	Mean	Geo Mean	CY	Median	Peak Ch
All	10000	100.00	100.00	6.77	5.17	87.60	4.87	1
M1	5	0.05	0.05	72.10	64.94	59.60	52.33	48

B.

PKH67.001

Counts / PKH67 FITC

File: PKH67.001
Gate: No Gate

Marker	Events%	Gated%	Total	Mean	Geo Mean	CY	Median	Peak Ch
All	10000	100.00	100.00	906.43	421.11	1112.52	486.97	1485
M1	9216	92.16	92.16	982.05	571.76	104.63	562.34	1485

C.

PKH67.002

Counts / PKH67 FITC

File: PKH67.002
Gate: No Gate

Marker	Events%	Gated%	Total	Mean	Geo Mean	CY	Median	Peak Ch
All	10000	100.00	100.00	120.87	82.82	191.82	89.77	77
M1	8274	82.74	82.74	140.50	111.63	178.21	103.66	77

D.

PKH67.004

Counts / PKH67 FITC

File:PKH67.004
Gate:No Gate

Marker	Events%	Gated%	Total	Mean	Geo Mean	CY	Median	Peak Ch
All	10000	100.00	100.00	240.08	125.12	231.02	148.55	184
M1	7292	72.92	72.92	315.68	215.90	200.49	205.35	184

E.

PKH67.001

Counts / PKH67

File:PKH67.001

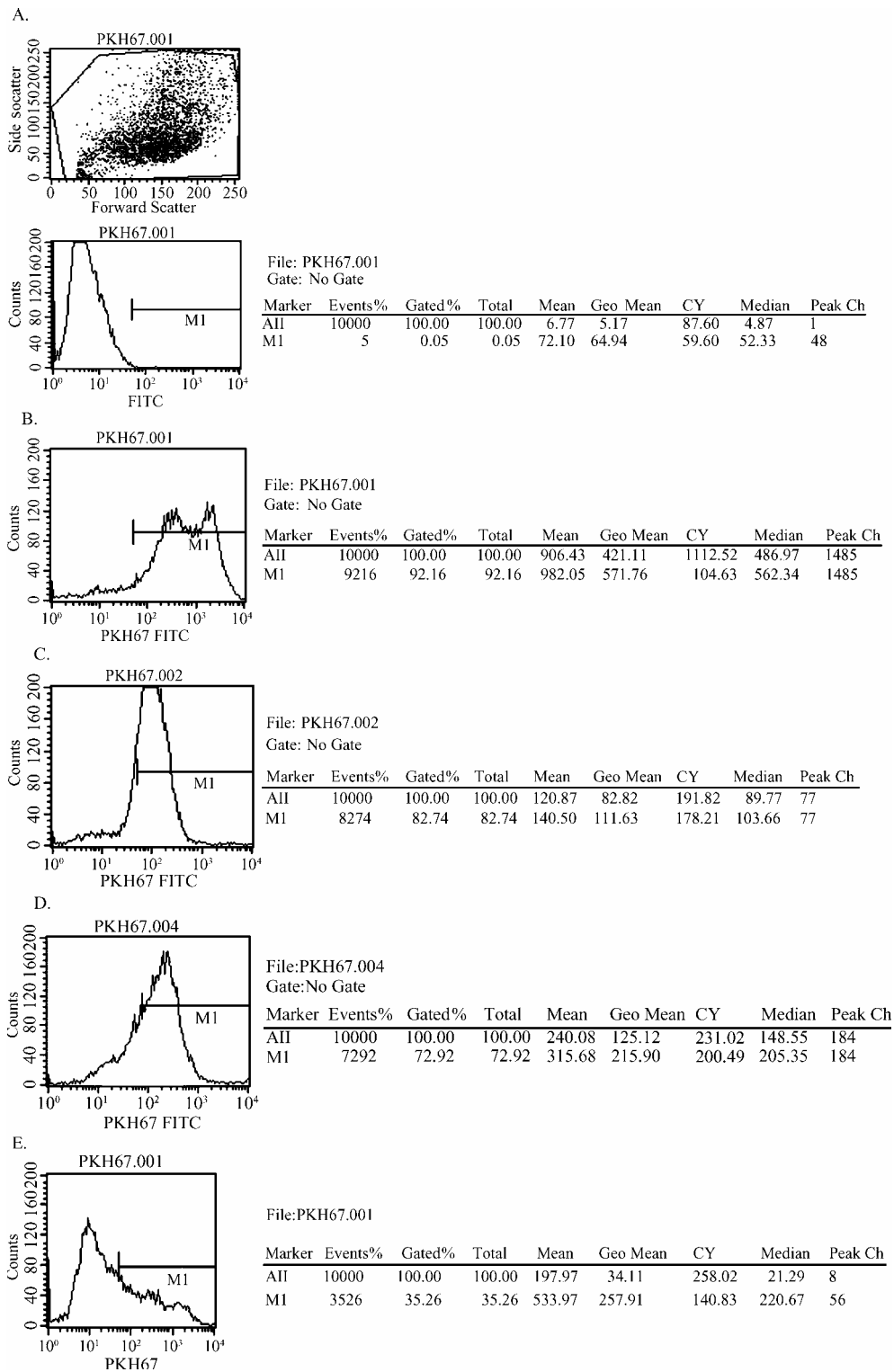| Marker	Events%	Gated%	Total	Mean	Geo Mean	CY	Median	Peak Ch
All	10000	100.00	100.00	197.97	34.11	258.02	21.29	8
M1	3526	35.26	35.26	533.97	257.91	140.83	220.67	56

FIG. 3. Flow cytometry analysis data shows the attenuation of green fluorescence of PKH67 labelled MSCs during the 3-week culture. The percentage of staining of PKH67 was 92.16% immediately after operation (B), 82.74% after 1 week (C), 72.92% after 2 weeks (D), and 82.74% after 3 weeks (E). (A) The negative control.
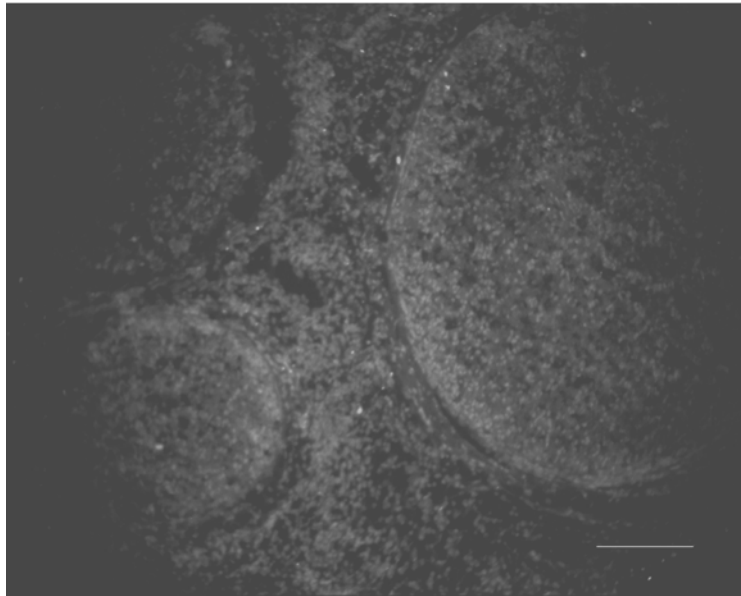
Fɪɢ. 4. Accumulation of PKH-67 labelled MSCs (Green) around the injured nerve fiber stem, and penetration of some MSCs into the adjacent normal nerve fiber stem two weeks after injection. (100×)

whether they could survive *in vivo* and whether they could adopt Schwann cell phenotype in injured peripheral nerve.

The PKH-67-GL cell linker kit uses patented membrane-labelling technology to stably incorporate a fluorescent dye with long aliphatic tails (PKH67) into lipid regions of the cell membrane. With reduced cell-cell transfer, PKH67 has been found to be useful for both *in vitro* cell proliferation labelling[17] and *in vivo* cell tracking for a relatively short time [18]. In our study, PKH67 labelled MSCs having adopted the phenotype of Schwann cells, expressed yellow fluorescence overlapped by green and red fluorescence, while those not expressing the glial-specific markers only expressed green fluorescence.

The artificial nerve conduit not only acts as a kind of shield to maintain the MSCs differentiation microenvironment of MSCs but also functions as a guide for regenerated nerve fiber orientation. The artificial conduit was made from de-acetyl chitin and can be degraded *in vivo*. In fact, we used it as an artificial nerve regeneration conduit combined with MSCs to promote the next step nerve regeneration. During our cell culture process, we found that without the addition of LIF, the differentiation potential of MSCs was partially lost after 4-6 population doublings. After adding the LIF during the cell culture following Yue-Hua JING's procedure[7], we achieved   satisfactory differentiation results.

The sequential administration of various factors such as β-ME, RA, followed by a mixture of FSK, bFGF, PDGF-AA and HRG effectively induces MSCs into cells with Schwann cell phenotype,

expressing GFAP,S-100, and P75. β-ME and RA are presumed to work as triggering factors, inducing changes in the morphological and transcriptional characteristics of MSCs. Sequential administration of β-ME and RA, followed by a mixture of FSK, bFGF, PDGF-AA and HRG has a cumulative effect on turning MSCs into Schwann cell phenotype.

During the *in vivo* cell tracing process, the fluorescence attenuation of PKH67 labelled MSCs was analyzed by flow cytometry every week after operation. The results revealed that PKH67 labeled MSCs amounted to 72.92% of total cell quantity after two weeks and the fluorescence intensity attenuated gradually. This fluorescence attenuation rule implied the comparative fluorescence attenuation *in vivo*.

Laser confocal microscopy co-localizes the green fluorescence of PKH67 labeled MSCs and the red fluorescence expressing of GFAP, S-100 and P75 with TRITC-conjugated Affinipure goat anti-rabbit Ab IgG (H+L). In our study,   only some co-localization of the PKH67 labelled MSCs without specific nerve fiber structure was found. This can be attributed to the nerve damage extent and a short time frame (only two weeks for nerve axon regeneration). The damaged sciatic nerve site had no continuous fibers and the regenerated axons were irregular at longitudinal sections two weeks after operation. The blue arrow in Fig. 8 points to the red fluorescence expressing axon transverse section with round morphology that was positive for P75. The 5 μm continuous frozen sections and the laser confocal microscopy excluded other non-specific color staining. Our data showed that MSCs injected into.
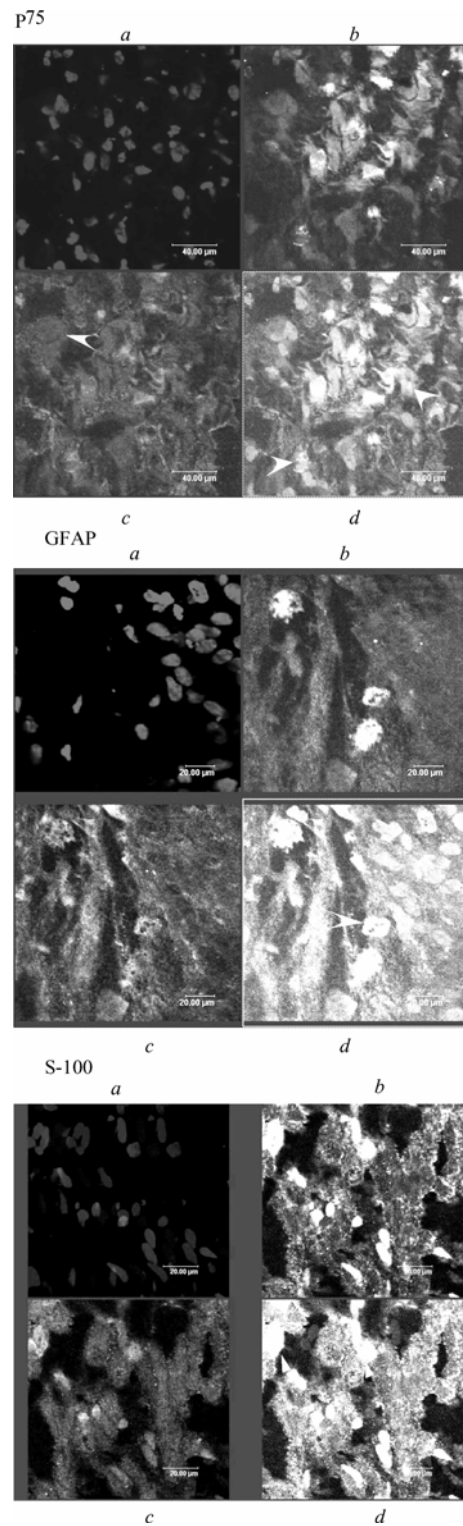
Fɪɢ. 5. Immunohistochemical staining of PKH67 labelled MSCs at the nerve-injured site two weeks after the transplantation. Green fluorescence (**b**) marks the transplanted MSCs labeled with PKH67. Red fluorescence (**c**) illustrates the location of the Schwann cell markers: GFAP, S-100, p75, respectively. The yellow spots (white arrowheads) at the double-labeled section (**d**) represent the PKH67 labeled MSCs that express the Schwann cell markers: GFAP, S-100, p75, respectively. DAPI counterstaining (**a**) illustrates cell nuclei for cell localization as contrast.

injured nerves could survive and adopt Schwann cell phenotype *in vivo*. In the present study, transplanted MSCs expressed GFAP, S-100 and P75, which are considered to be Schwann cell-specific markers.

Although cell fusion is the principle cell source after bone marrow stromal cell transplantation[19-20], our experimental results could not discriminate the cell differentiation phenomenon from cell fusion. Indeed, we did not find any cells with two nuclei in confocal figures. Regardless of mitosis or cell fusion, the green PKH-labeled, transplanted cells took on the Schwann cell phenotype and functioned as Schwann cells.

Marrow stromal cells are certainly one of the most attractive cell sources for neural transplantation because they exhibit several important and potentially advantageous features. They are easy to isolate from bone marrow and can readily expand under culture conditions. Therefore, differentiated MSCs are one of the strongest candidates for cell transplantation involving the nervous system.

In our study, only 5%-10% of MSCs were induced to Schwann cell like cells *in vitro*. Yet our experimental results provide the possibility of transformation *in vitro* and *in vivo*. More work should be done to increase the transformation rate so as to identify the other induced cell phenotypes.

ACKNOWLEDGEMENTS

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