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RESEARCH ARTICLE

Peripheral nerve regeneration using autologous porcine skin-derived mesenchymal stem cells

Bong-Wook Park[1#], Dong-Ho Kang[2#], Eun-Ju Kang[3], June-Ho Byun[1], Jong-Sil Lee[4], Geun-Ho Maeng[3] and Gyu-Jin Rho[3*]

[1]*Department of Oral and Maxillofacial Surgery, School of Medicine and Institute of Health Science, Gyeongsang National University, Jinju, Republic of Korea*
[2]*Department of Neurosurgery, School of Medicine, Gyeongsang National University, Jinju, Republic of Korea*
[3]*OBS/Theriogenology and Biotechnology, College of Veterinary Medicine and Research Institute of Life Science, Gyeongsang National University, Jinju, Republic of Korea*
[4]*Department of Pathology, School of Medicine, Gyeongsang National University, Jinju, Republic of Korea*

Abstract

Porcine skin-derived mesenchymal stem cells (pSMSCs) were evaluated on their biological MSC characterizations and differentiation into mesenchymal lineages, along with *in vitro* and *in vivo* neural inductions. Isolated pSMSCs showed plate-adherent growth, expression of various MSC-marker proteins and transcriptional factors, and differentiation potential into mesenchymal lineages. Neuron-like cell morphology and various neural markers were highly detected at 6 h and 24 h after *in vitro* neural induction of pSMSCs, but their neuron-like characteristics disappeared as induction time extended to 48 and 72 h. To evaluate the *in vivo* peripheral nerve regeneration potential of pSMSCs, a total of 5×10^6 autologous pSMSCs labelled with tracking dye, supplemented with fibrin glue scaffold and collagen tubulization, were transplanted into the peripheral nerve defected miniature pigs. At 2 and 4 weeks after cell transplantation, well-preserved transplanted cells and remarkable *in vivo* nerve regeneration, including histologically complete nerve bundles, were observed in the regenerated nerve tissues. Moreover, S-100 protein and p75 nerve growth factor receptor were more highly detected in regenerated nerve fibres compared to non-cell grafted control fibres. These results suggest that autologous pSMSCs transplanted with fibrin glue scaffold can induce prominent nerve regeneration in porcine peripheral nerve defect sites. Copyright © 2011 John Wiley & Sons, Ltd.

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1. Introduction

A range of techniques has been developed to improve nerve function in patients with damaged nervous systems. Autologous nerve grafts and the tubulization of injured nerves, including proper nerve guides, are the most popular peripheral nerve regeneration techniques (Patel *et al*., 2007). For this, various tissue-engineered cells or conduits have been used to enhance peripheral nerve regeneration. Cultured Schwann cells and neural stem cells improved axonal regeneration in peripheral nerve defect models (Heine *et al*., 2004; Mosahebi *et al*., 2001; Murakami *et al*., 2003). Recently, mesenchymal stem cells (MSCs) have generated great interest, due to their potential for self-renewal and rapid expansion in culture and being immunological inert and capable of long-term survival and differentiation into multi-lineages (Azizi *et al*., 1998; Rho *et al*., 2009; Tohill *et al*., 2004). Although effective generation and trans-differentiation *in vitro* into Schwann cell-like cells of

**Correspondence to: Gyu-Jin Rho, OBS/Theriogenology and Biotechnology, College of Veterinary Medicine, Gyeongsang National University, 900 Gajwa, Jinju 660-701, Republic of Korea. E-mail: jinrho@gnu.ac.kr*
[#]These two authors contributed equally to this work.

bone marrow-derived MSCs (BMSCs) has already been reported by several investigators (Cuevas *et al.*, 2004; Dezawa *et al.*, 2001; Hou *et al.*, 2006; Tohill *et al.*, 2004), bone marrow aspiration could involve invasive procedures and induce a range of complications in patients (Kang *et al.*, 2010). Recently, more accessible tissues, including fat or skin, have been investigated as alternative sources of adult stem cells for tissue engineering (Shi *et al.*, 2006).

Skin has been considered most recently as a potential adult stem cell source. It is highly accessible, and autologous tissue can be obtained easily with minimal donor site complications. Moreover, skin is an abundant pluripotent and multipotent cell source with an immune privilege and the potential for self-replication (Shi *et al.*, 2006; Toma *et al.*, 2005). It has been demonstrated that there are several different types of stem cells, such as skin-derived precursors (SKPs), skin-derived MSCs (SMSCs) and epidermal stem cells, in the dermis and epidermis of skins (Blanpain and Fuchs, 2006; Dyce *et al.*, 2004; Ohyama *et al.*, 2006; Riekstina *et al.*, 2008; Shi *et al.*, 2006).

Recently we isolated porcine skin-derived cells from the ear skin of miniature pigs and showed their MSC characters (Kang *et al.*, 2010). The cells were isolated from the epidermis and dermis of skin in serum-containing medium, and they proliferated adherently on the culture plate and expressed MSC-specific cell surface markers. In the present study, we reconfirmed the characters of these porcine skin-derived MSC-like cells (pSMSCs) with re-evaluation of transcription factors and cell surface marker proteins as well as mesenchymal lineage differentiation potential. Then we analysed *in vitro* neurogenic characteristics of pSMSCs during neuronal induction under a previously introduced chemical protocol (Woodbury *et al.*, 2002). Finally, as the main goal of this study, we investigated the *in vivo* peripheral nerve regeneration potential of autologous pSMSCs after transplantation into the femoral nerve defect model.

2. Materials and methods

2.1. Chemicals and media

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and media from Gibco (Invitrogen, Carlsbad, CA, USA). For all the media the pH was adjusted to 7.4 and the osmolality to 280 mOsm/kg.

2.2. Isolation and cultivation of pSMSCs

The animal model and isolation methods of pSMSCs in the present study were identical to those used in the previous study (Kang *et al.*, 2010). All experiments were authorized by the Animal Center for Medical Experimentation at Gyeongsang National University. Briefly, eight miniature pigs, aged 6 months–1 year and weighing approximately 25 kg, were sedated and anaesthetized using 4 mg/kg azaperone (Stresnil®; Janssen Animal Health for Merial Canada Inc., Baie d'Urfe, Quebec, Canada) and 10 mg/kg tiletamine-zolazepam (Zoletil®, Virbac, Carros, France). Under aseptic conditions, the ear skin samples of miniature pigs were obtained. After all hairs and subcutaneous fat tissues had been removed, the samples were cut into 1–3 mm^2 explants containing the epidermis and dermis. The explants were attached to the culture plates and 2 ml Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1), supplemented with 10% fetal bovine serum (FBS; Invitrogen), 10 ng/ml epidermal growth factor (EGF), 10 ng/ml basic fibroblast growth factor (bFGF), 100 U/ml penicillin and 100 μg/ml streptomycin were added, and the plates were incubated at 38.5 °C in a humidified atmosphere containing 5% CO_2 in air for 3 days. After the remaining skin fragments had been removed, the attached cells were further expanded *in vitro* by changing the culture medium twice a week. Once confluent, the cells were dissociated using a 0.25% w/v trypsin–ethylenediaminetetraacetic acid (EDTA; Invitrogen) solution and pelleted at $500 \times g$ for 5 min. The cells were then grown further until the passage 3.

2.3. Characterization of isolated pSMSCs

To investigate the expression of MSC markers (CD29, CD44, CD90 and vimentin) and immune reaction activity (swine leukocyte antigen, SLA) of pSMSCs, cells at passage 3 were analysed by flow cytometer (BD FACSCalibur; Becton Dickinson, Franklin Lakes, NJ, USA) in thee replicates. In brief, cells that reached 90% confluence were harvested using 0.25% EDTA and washed twice in Dulbecco's phosphate-buffered saline (DPBS) supplemented with 10% FBS. To detect CD44 and CD90, cells were directly labelled with fluorescein isothiocyanate (FITC)-conjugated CD markers. However, to analyse CD29, vimentin and SLA, cells were fixed in 3.7% formaldehyde for 1 h, washed with DPBS and sequentially labelled with primary and secondary antibodies for 1 h. All antibodies were diluted to 1:100 and the detailed information of antibodies for fluorescence-activated cell sorting (FACS) analysis is summarized in Table 1.

pSMSCs at passage 3 were evaluated for the expression of transcription factors, such as *Oct-4, Sox-2* and *Nanog*, by reverse transcription–polymerase chain reaction (RT–PCR) and immunocytochemistry. Total RNA extraction, cDNA synthesis and the RT–PCR procedure of the present study were performed according to previously described protocols (Kang *et al.*, 2010) and the PCR primers are summarized in Table 2. The antibodies and dilution information regarding immunocytochemical staining of pSMSCs for transcription factors are summarized in Table 3.

To assess *in vitro* mesenchymal lineage differentiation, pSMSCs at passage 3 were seeded at a density of 1×10^4

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Table 1. Primary and secondary antibodies for fluorescence-activated cell sorting (FACS) analysis of pSMSCs

Name	Type	Company	Amount (mg/ml)	Dilution
Mouse anti-pig CD29	IgG1	BD Pharmingen	0.5	1:100
FITC anti-mouse CD44	IgG2b	BD Pharmingen	0.5	1:100
FITC anti-human CD90	IgG1	BD Pharmingen	0.5	1:100
Mouse anti-vimentin	Monoclonal IgG1	Sigma-Aldrich	0.5	1:100
Mouse anti-pig SLA-DR	IgG2a	BD Pharmingen	0.5	1:100
FITC goat anti-mouse IgG	Goat polyclonal	BD Pharmingen	0.5	1:100
FITC mouse isotype control	Polyclonal IgG1	BD Pharmingen	0.5	1:100

Companies: BD Pharmingen™, BD Biosciences, Franklin Lake, NJ, USA; Sigma-Aldrich, St. Louis, MO, USA.

Table 2. RT–PCR primers used for evaluating transcription factors in the pSMSCs

Gene	Sequence of primer (5′–3′)	Product size (bp)	Annealing T_m (°C)
GAPDH*	F, GGGCATGAACCATGAGAAGT R, AAGCAGGGATGATGTTCTGG	230	60
Oct4	F, AGGTGTTCAGCCAAACGACC R, TGATCGTTTGCCCTTCTGGC	335	60
Nanog	F, ATCCAGCTTGTCCCCAAAG R, ATTTCATTCGCTGGTTCTGG	438	60
Sox2	F, GCCTGGGCGCCGAGTGGA R, GGGCGAGCCGTTCATGTAGGTCTG	443	64

*GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

cell/cm^2 in the specific conductive conditions following previously reported protocols with minor modifications (Pittenger *et al*., 1999; Vacanti *et al*., 2005). Osteogenic differentiation was inducted in DMEM with 10% FBS, 0.1 μM dexamethasone, 50 μM ascorbate 2-phosphate and 10 mM β-glycerol phosphate for 4 weeks. To confirm calcium accumulation, differentiated cells were fixed in 3.7% formaldehyde, followed by von Kossa staining with 5% $AgNO_3$ in the light for 1 h at room temperature. Adipogenic differentiation was inducted in DMEM with 10% FBS, 1 μM dexamethasone, 10 μM insulin and 200 μM indomethacin for 4 weeks. For the evaluation of intracellular accumulation of lipids, cells were incubated for 10 min with oil red O solution. Osteogenic and adipogenic differentiated cells for 4 weeks were analysed for expressions of bone [osteonectin (ON) and osteocalcin (OC)] and adipocyte [peroxisome proliferators-activated receptor-γ2 (PPARγ2) and adipocyte fatty acid-binding protein (aP2)]-specific marker proteins by RT–PCR, respectively. Chondrogenesis was induced with chondrocyte differentiation medium (HyClone Advance STEM™, Thermo Fisher Scientific Inc., Waltham, MA, USA) for 4 weeks. To observe the presence of proteoglycans, cells were fixed with 3.7% formaldehyde, followed by staining with 1% alcian blue dissolved in 3% acetic acid for 30 min at room temperature.

2.4. *In vitro* neuronal differentiation of pSMSCs

Neuronal differentiation of pSMSCs was performed using a slightly modified protocol described by Woodbury and colleagues (2002). Briefly, pSMSCs at 70% confluence were cultured in neuronal preinduction medium containing DMEM with 20% FBS and 10 ng/ml bFGF for 24 h. The cells were then cultured in neuronal induction medium composed of DMEM supplemented with 2% dimethylsulphoxide (DMSO), 200 μM butylated hydroxyanisole (BHA), 25 mM KCl, 2 mM valproic acid, 10 μM forskolin, 1 μM hydrocortisone, 5 μg/ml insulin and 2 mM L-glutamine without FBS for up to 48 h in order to initiate *in vitro* neural differentiation.

2.5. Immunocytochemical analysis of *in vitro* neural differentiation cells

Cells were fixed at 0, 6 and 48 h after incubation in neuronal induction medium with 3.7% formaldehyde solution for 30 min. After being permeabilized and blocked in 0.1% Triton X-100 solution containing 1% bovine serum albumin (BSA) for 1 h, the cells were incubated with the primary antibodies: neural precursor marker (nestin); high- and low-affinity nerve growth factor receptors (TrkA and p75NGFR); neuron related microtubule (β-tubulin III); neuron-specific intermediate filament (neurofilament, NF); neural crest-originated cell marker protein (S-100); and dopamine precursor-related enzyme (tyrosine hydroxylase, TH), at 4°C overnight. After being rinsed three times with DPBS, the cells were incubated with FITC-conjugated secondary antibodies for 45 min at 37°C. The slides with cells were mounted with Vectashield® (Vector Laboratories, CA, USA). Images were observed under fluorescence microscope (Leica CTR600, Switzerland). Mean intensity was measured by analySIS TS™ software (Olympus Soft Imaging Solutions, Münster, Germany). For evaluation

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Table 3. Primary and secondary antibodies of the immunocytochemical studies for the expression of transcription factors in pSMSCs and various nerve-related markers in *in vitro* neural inducted cells

Name	Type	Company	Amount	Dilution
Oct 4	Goat polyclonal	Santa Cruz Biotechnology	200 μg/ml	1:100
Nanog	Goat polyclonal	Santa Cruz Biotechnology	200 μg/ml	1:100
Sox 2	Rabbit polyclonal	Santa Cruz Biotechnology	200 μg/ml	1:100
β-Tubulin III	Goat polyclonal	Santa Cruz Biotechnology	200 μg/ml	1:100
Trk A	Goat polyclonal	Santa Cruz Biotechnology	200 μg/ml	1:100
S-100	Rabbit polyclonal	Thermo Fisher Scientific	0.1 ml	1:100
Neurofilament	Goat polyclonal	Santa Cruz Biotechnology	200 μg/ml	1:100
p75NGFR	Mouse momoclonal	Santa Cruz Biotechnology	200 μg/ml	1:100
Tyrosine hydroxylase	Rabbit polyclonal	Pel-Freez®	150 μl	1:1000
Nestin	Mouse monoclonal	BD Pharmingen™	0.5 mg/ml	1:200
FITC Donkey anti-goat IgG	Goat polyclonal	Jackson ImmunoResearch	1.5 mg/ml	1:200
FITC Donkey anti-rabbit IgG	Goat polyclonal	Jackson ImmunoResearch	1.5 mg/ml	1:200
FITC Goat anti-mouse Ig	Goat polyclonal	BD Pharmingen™	0.5 mg/ml	1:100

p75NGFR, p75 nerve growth factor receptor; trkA, tyrosine kinase receptor A.

Companies: Santa Cruz Biotechnology, Santa Cruz, CA, USA; Thermo Fisher Scientific Inc., Waltham, MA, USA; Pel-Freez®, Pel-Freez Biologicals, Rogers, AR, USA; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA.

of one antibody's expression, a minimum of three slides were immunostained and statistically analysed at each time point. Detailed information of the primary and secondary antibodies used for evaluation of *in vitro* neural differentiation of pSMSCs is given in Table 3.

2.6. *In vivo* transplantation of autologous pSMSCs in the peripheral nerve defect sites

Once they had attained 90% confluence, pSMSCs were labelled with the fluorescent lipophilic carbocyanine dye PKH26 (Sigma-Aldrich) according to the manufacturer's instructions. The unilateral femoral nerve from four experimental animals was exposed after sequential dissection of the lateral thigh under general anaesthesia with a combination of azaperone and tiletamine–zolazepam. The exposed femoral nerves were resected into the 10 mm gap, and both resected nerve ends were covered and enveloped with biodegradable bovine collagen dura mater (Lyoplant®; Aesculap, Melsungen, Germany) and 6-0 silk suture material. This process formed a tubule in the resected nerve gap. A total of 5×10^6 autologous pSMSCs labelled with PKH26 were transplanted into the resected nerve gap with 0.3 ml fibrin glue (Greenplast® kit, Green Cross, Yongin, Korea) in the experimental group (Figure 5A, B), followed by being sutured to seal the resorbable membrane (Lyoplant®) tubule. In the four control animals, only fibrin glue scaffold was injected into the femoral nerve gap.

2.7. Histological analysis of *in vivo* peripheral nerve regeneration

At 2 and 4 weeks after autologous pSMSCs transplantation in the nerve gaps, each two animals (transplanted and control) at the time points were euthanized by KCl injection under general anaesthesia. The resected femoral nerves were re-exposed and harvested *en bloc* and the specimens were carefully divided into longitudinal sections for histological studies and fluorescence analysis.

Some of the specimens were used for observation of PKH26-stained cells in a manner similar to that of the previous study (Kang *et al*., 2010). Briefly, tissues were embedded in optimum cutting temperature (OCT) compound (Tissue-Tek®; Sakura Finetechnical Co. Ltd, Tokyo, Japan), rapidly frozen at −23 °C and cut into 4 μm sections using Cryocut equipment (Leica CM3050S; Leica, Wetzlar, Germany). The sections were mounted on glass slides and counterstained with 4′,6-diamino-2-phenylindole (DAPI; Vectasheid®, Vector, Burlingame, CA, USA). Fluorescent expression was assessed by fluorescence microscopy (BX51, Olympus, Tokyo, Japan), using a fluorescent digital camera (DP72; Olympus). The ratio of PKH26-stained to DAPI-stained cells was calculated in each *in vivo* specimen. A minimum of four glass slides of each grafted group were evaluated.

The remnant specimens were fixed with 10% neutral buffered formalin for 24 h and embedded in a paraffin block, cut into 4 μm sections and then mounted on silane-coated slides. The sections were maintained at room temperature for 12 h and deparaffinized. Haematoxylin and eosin (H&E) staining was conducted after hydration. Immunohistochemical staining for S-100 protein and p75 nerve growth factor receptor (p75NGFR) was conducted using an automated immunostainer (LabVision Autostainer™; LabVision, Thermo Fisher Scientific Inc., Fremont, CA, USA). Deparaffinization and antigen retrieval of specimens were performed simultaneously using Tris–EDTA buffer (LabVision). Glass slides were incubated in a PTmodule™ (LabVision) at 100 °C for 25 min, washed with Tris-buffered saline (LabVision) twice (3 min each time) and treated with hydrogen peroxide at room temperature for

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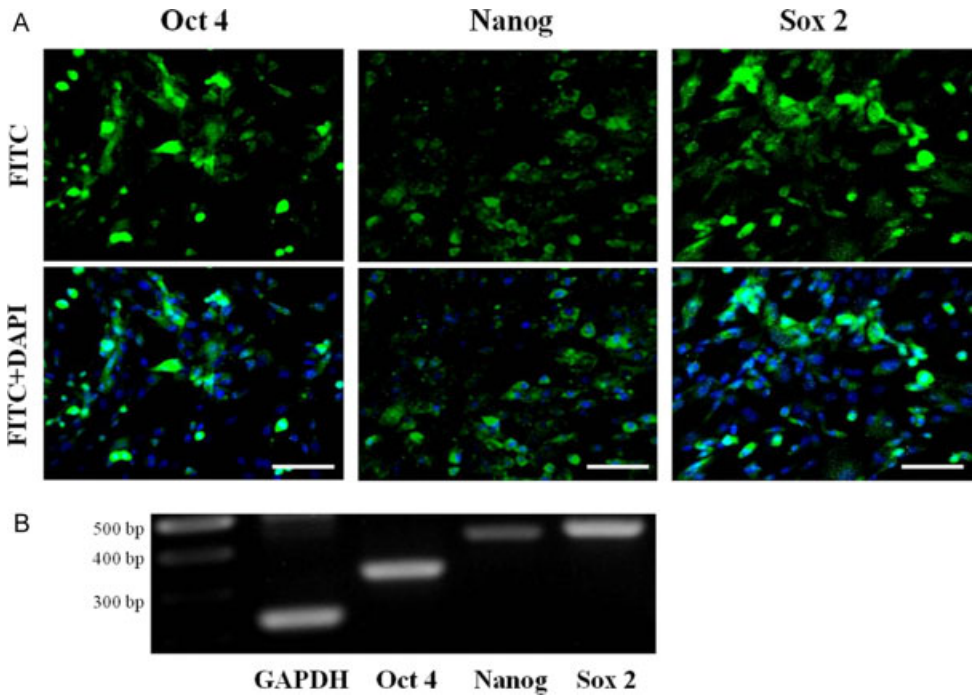

Figure 1. Expression of transcription factors, Oct 4, Nanog and Sox 2, in cultivated pSMSCs at passage 3 by immunocytochemistry (A) and RT-PCR (B) (green, FITC; blue, DAPI; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; scale bar = 100 μm). All these all early transcription factors were detected at the protein and mRNA levels of the pSMSCs

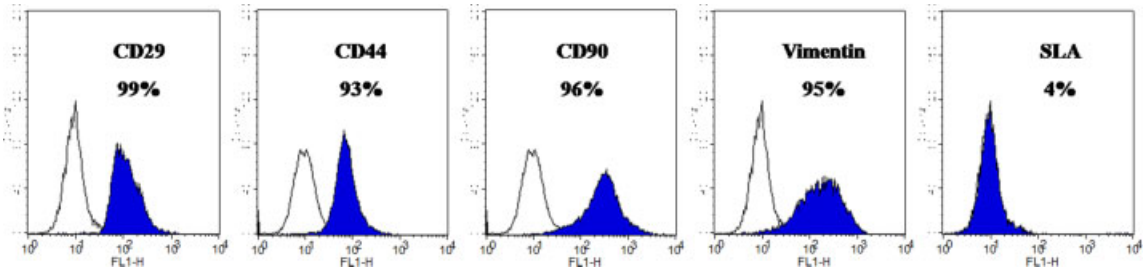

Figure 2. Fluorescence-activated cell sorting (FACS) analysis of MSC marker proteins and swine leukocyte antigen (SLA) in pSMSCs of passage 3. The MSC markers CD29, CD44, CD90 and vimentin, were highly detected in our isolated pSMSCs, whereas immune response-related antigen (SLA) was almost negatively detected. Open histograms represent staining with negative control, and the filled histograms represent the fluorescence intensity of each cell surface antibody

10 min. A 1:200 dilution of primary rabbit polyclonal antibody IgG (S-100; RB-9018, LabVision, Thermo Fisher Scientific) and a 1:200 dilution of primary mouse monoclonal antibody IgG (p75NGFR; sc-13 577, Santa Cruz) were used to observe S-100 and p75NGFR expression in the regenerated nerve tissue. The sections were exposed to primary antibodies against S-100 and p75NGFR at room temperature for 40 min, treated with a biotinylated polyvalent secondary antibody solution, incubated with a horseradish peroxidase-conjugated avidin–biotin complex and treated with 3,3-diaminobenzidine and hydrogen peroxidase. Finally, the nuclei were counterstained with haematoxylin. Densitometric and morphometric analyses of p75NGFR expressions were performed using analySIS TS™ software (Olympus Soft Imaging Solutions) in each specimen; the areas were presented as μm^2. To analyse the *in vivo* expression intensity of p75NGFR, highly expressed sites were selected in each transplanted specimen.

2.8. Statistical analysis

All values of the present study were statistically analysed by one-way ANOVA and independent grouping variables were compared using Bonferroni and SPSS software. Data were expressed as a mean ± SD. Differences were considered to be significant when $p < 0.05$.

3. Results

3.1. Isolation and characterization of pSMSCs

In the primary culture, porcine skin-derived cells from the ear skin were grown into a population of fibroblast-like adherent cells and showed heterogeneously irregular shapes. After a week in the primary culture, the cells were characterized by forming partially distributed colonies. By

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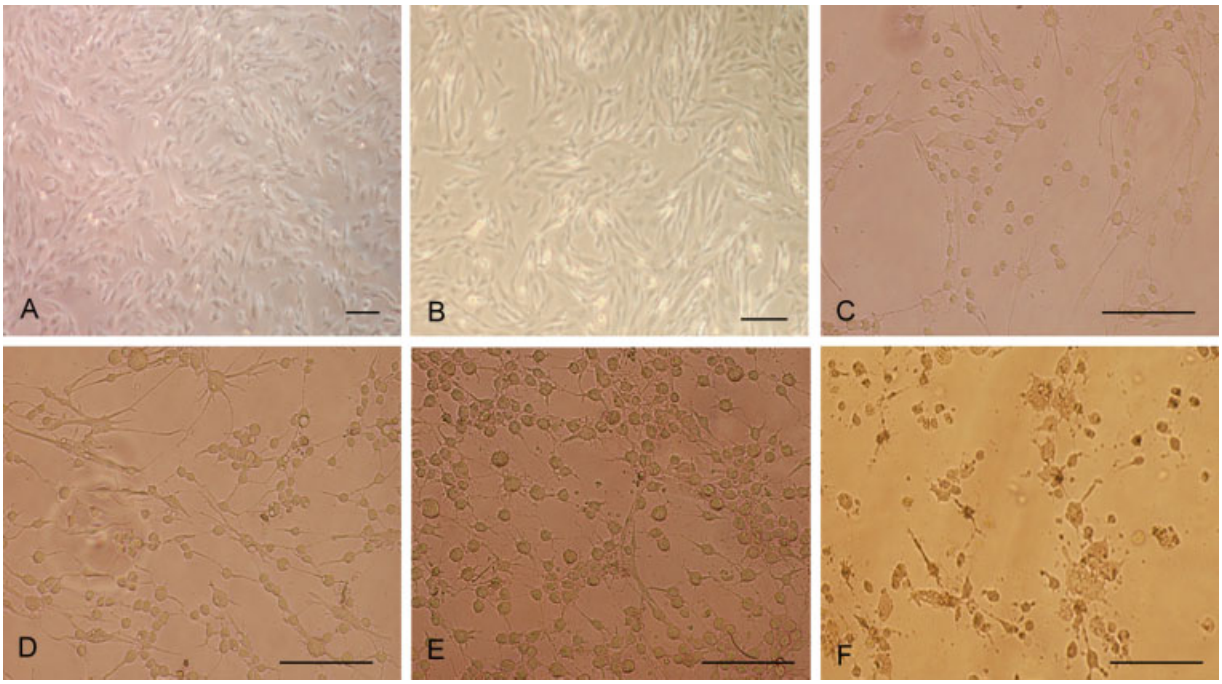

Figure 3. pSMSCs at passage thee were differentiated *in vitro* to neuron-like cells under a chemical protocol (scale bar = 50 μm). (A) Microscopic features of incubated pSMSCs at passage 3. (B) Immediately after neuronal pre-induction for 24 h. No remarkable morphological change was detected at this stage. (C, D, E, F) Microphotographs at 3 (C), 6 (D), 24 (E) and 72 h (F) after chemical induction of pSMSCs. (C) Neuron-like morphological changes were detected from 3 h post-application of induction chemicals. (D, E) These neuron-like cells were more remarkable at 6 and 24 h after induction. (F) However, degradation of neuron-like morphology and decrease of cell number were observed as neuronal induction time progressed to 72 h

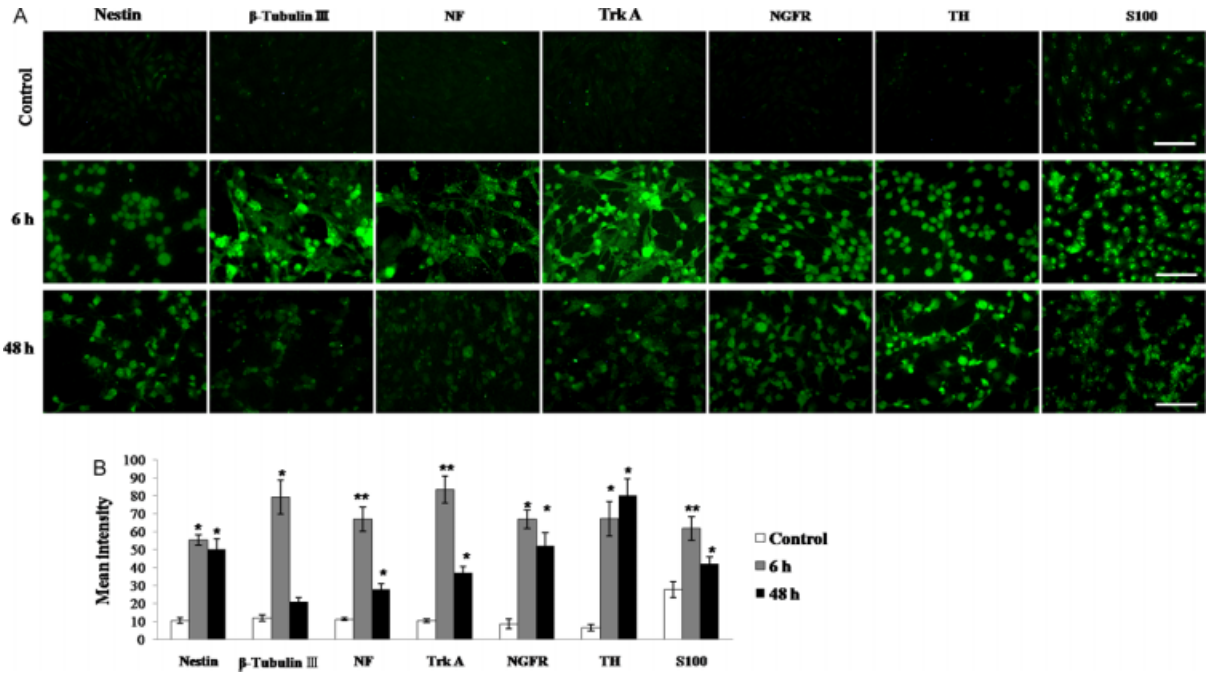

Figure 4. Expression patterns (A) and image intensities (B) of the immunocytochemical studies for various nerve cell-related markers during *in vitro* neuronal induction of pSMSCs (Control, non-inducted pSMSCs; 6 h, 6 h post-induction; 48 h, 48 h post-induction; scale bar = 50 μm). Most marker proteins were actively expressed at 6 h after chemical neuronal induction. However, the expression intensities decreased as induction time increased to 48 h (NF, neurofilament; TrkA, tyrosine kinase receptor A; NGFR, p75NGFR; TH, tyrosine hydroxylase; S100, S-100 protein)

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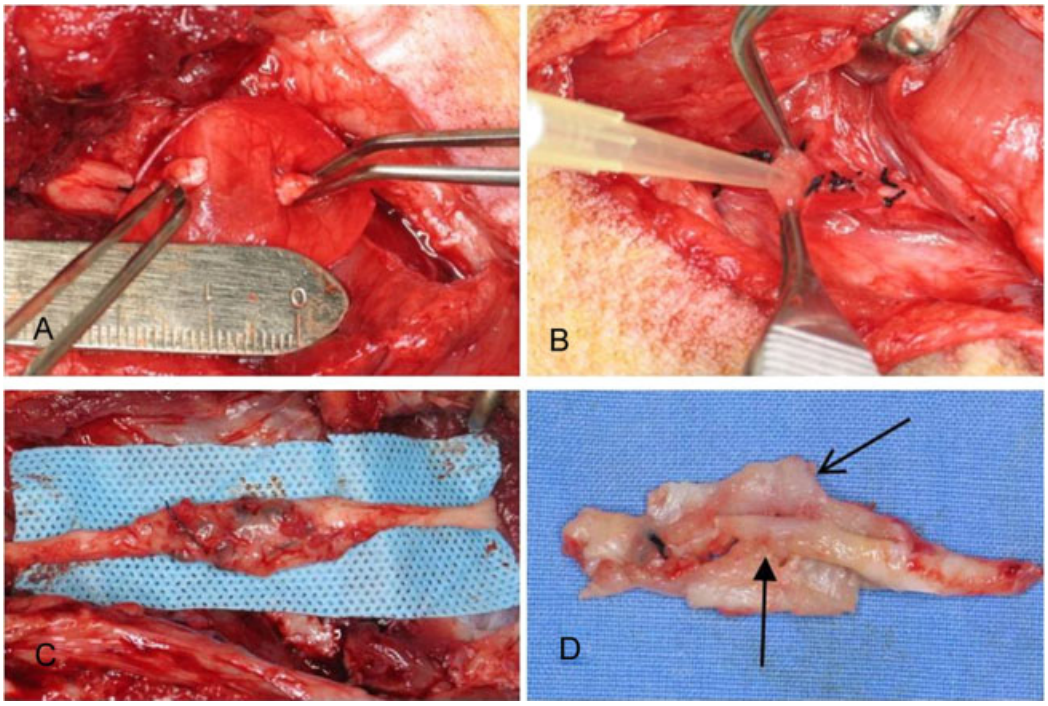

Figure 5. Schematic photographs of *in vivo* transplantation of autologous porcine SMSCs (pSMSCs) with fibrin glue scaffold in the femoral nerve defect model in the miniature pig. (A) Femoral nerve was dissected and cut to give a 10 mm gap. (B) Tubulization with biodegradable bovine collagen dura mater (Lyoplant®) was formed over the nerve gap, and 5×10^6 autologous pSMSCs with 0.3 ml fibrin glue scaffold (Greenplast® kit) were transplanted into the resected nerve gap. (C) Redissected appearance of the experimental nerve at 4 weeks after cell transplantation. Hypertrophic scar formation was usually observed in the cell transplanted nerve site. (D) Under the scar tissues and nerve tubule (open arrowhead), completely regenerated nerve continuity was observed (closed arrowhead)

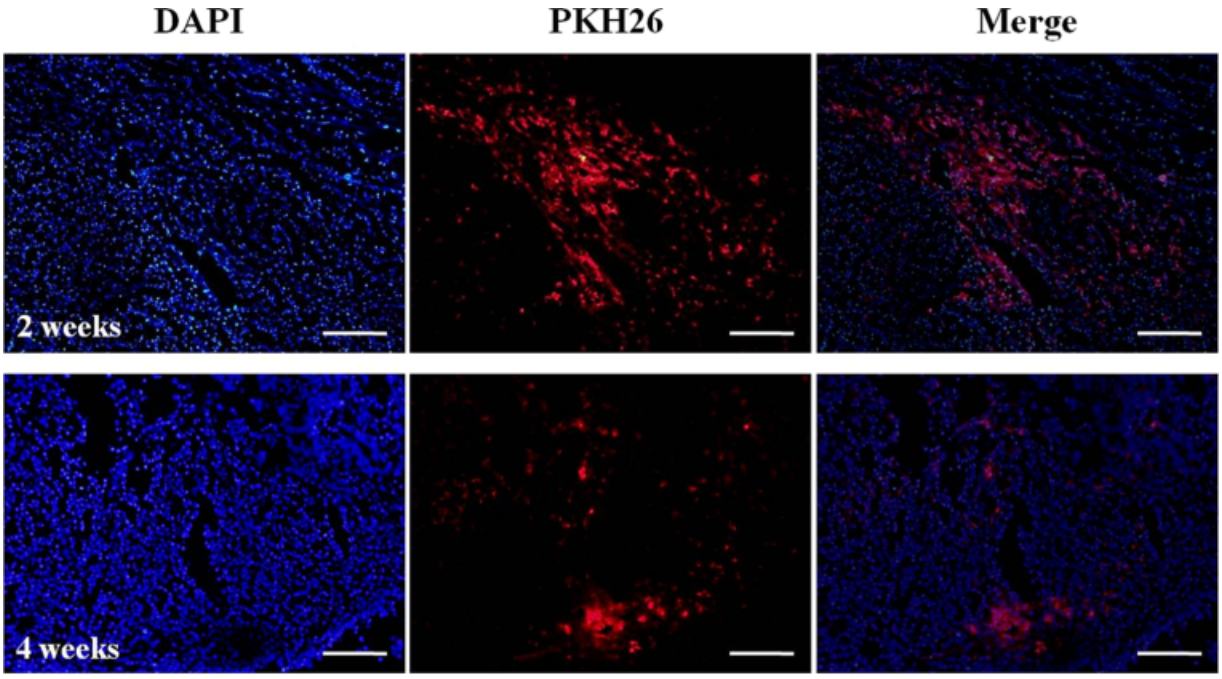

Figure 6. Detection of cell tracking dye, PKH26, in the autologous pSMSCs implanted sites (scale bar = 100 μm). In specimens both 2 and 4 weeks after *in vivo* transplantation, PKH26-positive cells were easily detected and well preserved in the regenerated experimental specimens

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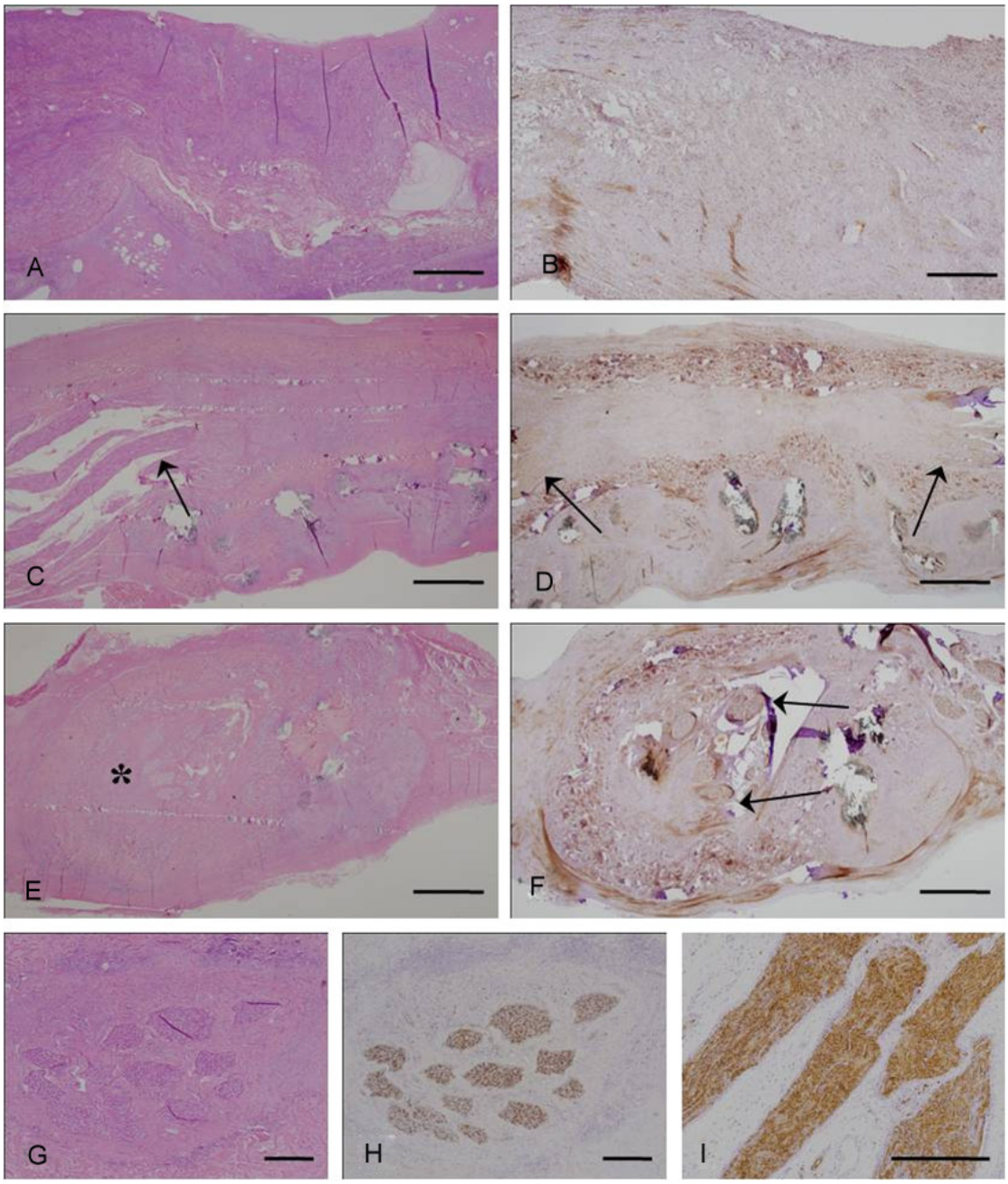

Figure 7. Histological features of the *in vivo* transplanted nerve specimens (A, C, E, G) H&E-stained; (B, D, F, H, I) S-100 stained; scale bar = 1000 μm (A–F); 100 μm (G–I). (A, B) Control specimens after 4 weeks of fibrin glue scaffold transplantation. Although nerve continuity was produced, neither the newly generated nerve fibre nor the expression of S-100 protein was appreciably detected in the reconstructed tissue. (C, D) The experimental specimens 2 weeks after autologous pSMSCs and fibrin glue scaffold transplantation. (C) Actively regrowing nerve fibres from the proximal ends of resected nerves were detected in the regenerated tissue (arrow). (D) At this time point, S-100 protein was more definitely detected in the experimental specimens than in the 4 weeks control specimen. Both proximal and distal nerve ends were observed in this specimen (both arrows). The distance between the two ends was shorter (about 5 mm) than that at the initial surgery (10 mm). (E, F) Experimental specimens 4 weeks after transplantation. (E) At this time point, hypertrophic healed tissues were easily found in the cell-transplanted segments, and the regenerated nerve fibres were also remarkably detected in these healed tissues (*). (F) The regenerated nerve fibres were clearly observed in the S-100 stained specimen (arrows). (G, H, I) Higher magnifications of the 4 weeks experimental specimen. Newly generated nerve bundles, including epineurium and perineurium and axonal fibres, were well detected in cross-sections (G, H) and longitudinal sections (I) of regenerated nerves

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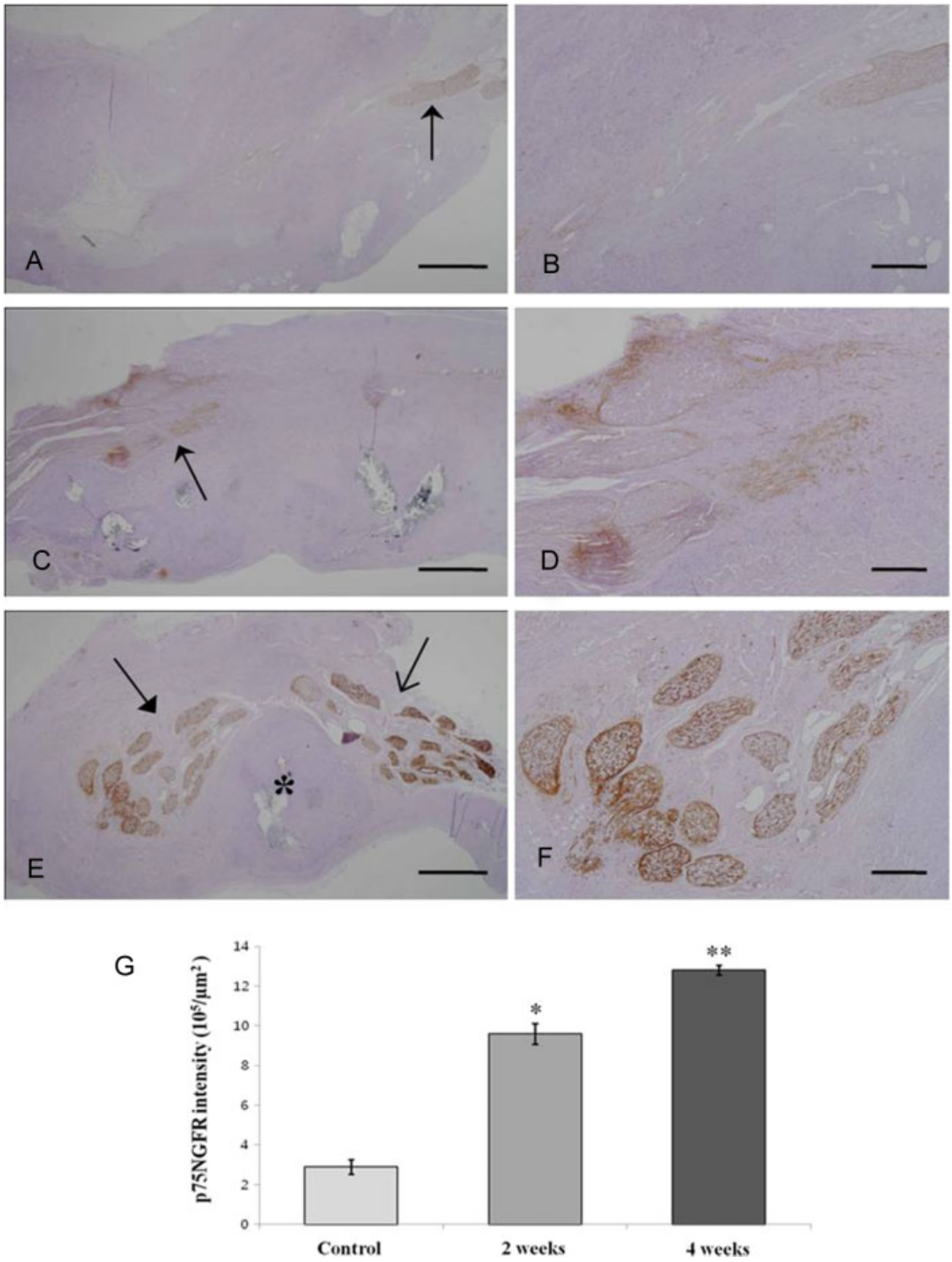

Figure 8. p75NGFR expression in the control and experimental specimens: scale bar = 1000 μm (A, C, E); 100 μm (B, D, F). (A, B) The control specimens of 4 weeks after scaffold transplantation. Weak expression of p75NGFR was detected in the peripheral portion of regenerated tissue (arrow). (C, D) The experimental specimens 2 weeks after cell transplantation showed strong expression of p75NGFR in the proximal part of regenerated nerve fibres (arrow). (E, F) The experimental specimens 4 weeks after cell transplantation exhibited more enhanced regeneration of nerve fibres and p75NGFR expression in the proximal (closed arrow) and distal (open arrow) portions of the regenerated tissues. Overgrown interstitial tissues were usually observed at this time point in the experimental specimens (*). (G) In image intensities of p75NGFR, more enhanced p75NGFR expression was observed in the autologous pSMSC-grafted nerve fibres than in the control fibres, and it was most substantially expressed in the 4 weeks experimental specimens

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14–21 days, the cells exhibited a homogeneous layer of adherent cells with the majority of colony formation.

The cells at passage three expressed transcription factors such as *Oct-4, Nanog* and *Sox-2*, according to immunofluorescence and RT–PCR analyses (Figure 1). In addition, the cells were positive for typical mesenchymal stem cell markers (CD29, CD44, CD90 and vimentin), according to FACS analysis, but did not express SLA, which is important for immune reaction (Figure 2). These results demonstrate that porcine skin-derived cells in the present study can be regarded as multipotent porcine skin-derived mesenchymal stem cells (pSMSCs), which have low immunity. In addition, *in vitro* differentiation of pSMSCs to mesenchymal lineages, including osteocytes, adipocytes and chondrocytes, were successfully induced in their specific media (data not shown).

3.2. *In vitro* neuronal induction of pSMSCs

No specific morphological changes from pSMSCs were observed after 24 h of neural pre-induction (Figure 3A, B). However, under conditions conducive to neural differentiation, pSMSCs were grown into a population of neuron-like adherent cells with retraction of cell body and process elaboration from 3 h after induction (Figure 3C). These neuron-like cell morphologies were remarkable as induction time extended to 6 and 24 h (Figure 3D, E). However, as induction time increased to over 48 and 72 h, the differentiated neuron-like cells decreased in cell number and their morphology degraded (Figure 3F).

In the immunocytochemical studies of *in vitro* differentiated neuron-like cells, nerve-related proteins, such as nestin, TrkA, p75NGFR, β-tubulin III, NF, S-100 and TH, were highly expressed and peaked at 6 h post-induction compared to non-inducted control pSMSCs. However, the expression intensity of neuron-related proteins, except for TH, decreased as induction time extended to 48 h (Figure 4).

3.3. *In vivo* transplantation of autologous pSMSCs into the resected peripheral nerve

On gross inspection at 4 weeks after *in vivo* autologous pSMSCs transplantation, the continuity of the resected nerve was obviously regenerated under the bovine collagen dura mater (Lyoplant®) tubule (Figure 5C, D). In addition, pSMSCs labelled with PKH26 were observed in the regenerated nerve tissues at 2 and 4 weeks after cell transplantation (Figure 6). The ratio of PKH26 stained cells to all observed (DAPI positive) cells was higher in the 2 weeks specimen than in the 4 weeks specimen (0.48 ± 0.06 and 0.16 ± 0.06 at 4 weeks, respectively; $p < 0.05$).

Optical microscopy with H&E staining demonstrated that the resected gap was filled with loose and dense connective tissues, and continuity was achieved in all specimens. In a control specimen weeks after transplantation, newly generating nerve fibres were hardly detected, except at the periphery of resected nerve ends (Figure 7A, B). However, in experimental specimens, nerve regeneration markedly increased in the specimens at 2 and 4 weeks after transplantatiopn (Figure 7C–F). In particular, nerve regeneration was greatly enhanced and formed complete nerve bundles in the resected nerve defect at 4 weeks after transplantation (Figure 7G–I). S-100 and p75NGFR were more strongly expressed in the experimental specimens than in the control (Figures 7, 8). In addition, p75NGFR was more specifically expressed in regenerated nerve fibres, and its intensity was strongest in the 4 weeks after cell transplantation group (Figure 8G).

4. Discussion

pSMSCs in the present study showed plate-adherent growing capacity, expression of MSC-specific cell surface markers and differentiation potential to mesenchymal lineages. In addition, the pSMSCs expressed the transcription factors *Oct4, Nanog* and *Sox2*, not only at the mRNA level according to RT–PCR but also at the protein level according to immunocytochemistry. These results indicate that pSMSCs are primitive MSC-characterized cells possessing multipotency. Interestingly, swine leukocyte antigen (SLA) was almost negatively detected in pSMSCs by FACS analysis, indicating that they may have low-immune reactions when they are transplanted (Lunney, 1994). In the present study, the *in vitro* neuronal differentiation of pSMSCs using a chemical induction protocol revealed the expression of morphological neuron-like cells and nerve-specific proteins within a few hours. These results were similar to previous studies, observations of nerve-like cell differentiation of BMSCs using similar protocols (Woodbury *et al.*, 2000, 2002). Although other *in vitro* neuronal differentiation methods of BMSCs, such as culture in nerve-related molecules (Kim *et al.*, 2002; Sanchez-Ramos *et al.*, 2002) or inflammatory astrocyte-containing medium (Wang *et al.*, 2009), and increasing intracellular cyclic AMP (Deng *et al.*, 2001) have been introduced, the chemical protocol method has been broadly used and studied. It was a simple approach that rapidly yielded neuron-like cell differentiation from BMSCs (Bossolasco *et al.*, 2005; Lei *et al.*, 2007; Yamaguchi *et al.*, 2006). In addition, >70% of differentiated neuron-like cells were detected after chemical induction of BMSCs, which was a very much higher concentration than in the other induction method (Barnabé *et al.*, 2009; Munoz-Elias *et al.*, 2003).

However, several researchers have reported that chemical compounds in serum-deprived conditions produced toxic and stressful conditions, which resulted in rapid disruption of the actin cytoskeleton and shape change to a form resembling nerve cells (Lu *et al.*, 2004; Neuhuber *et al.*, 2004). In addition, these chemically induced nerve-like cells usually showed increasing cell apoptosis or death, and did not exhibit electrical conductions (Barnabé *et al.*, 2009; Rismanchi *et al.*, 2003). Similarly, we also observed rapid cell morphological changes

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and nerve-related protein expression, although the MSC source of the present study was not bone marrow; however, these differentiated neuronal characters disappeared as induction time increased beyond 48 h. Interestingly, when the induction medium was changed to DMEM with FBS at 72 h post-induction, the neural differentiated cells returned to their pre-inducted shape and the cell number also increased (data not shown). These results indicate that the chemically induced neuron-like cells of the present study could not be regarded as true nerve cells. When stem cells differentiate to target cells, it is difficult for them to return to stem cells, especially in a short time period (Rho *et al*., 2009). Therefore, the chemical induction protocol was not a suitable choice for the neural differentiation method for pSMSCs, even though various nerve-related proteins were observed shortly after induction. However, these results did not demonstrate that pSMSCs cannot differentiate to neural cells. Yamaguchi *et al*. (2006) observed that BMSCs changed gene expression after treatment with chemical agents, and they suggested that this shift might be related to the multipotency of BMSCs. Similarly, in the present study, the results of *in vitro* chemical induction of pSMSCs indicate that they have a potential to change their characteristics in response to external stimuli.

A mere scaffold graft, such as a collagen filament, has shown an effective result in nerve regeneration (Yoshii *et al*., 2009). Moreover, many researchers have demonstrated enhanced *in vivo* nerve regeneration after transplantation of the differentiated Schwann cells or neurons from BMSCs, not only from stem cells (Dezawa, 2008; Shimizu *et al*., 2007). However, it is well known that undifferentiated MSCs have a potential for differentiation into target cells after *in vivo* transplantation (Rho *et al*., 2009). Moreover, undifferentiated MSCs have expressed various neurotrophic factors after *in vivo* transplantation into the defected nerve (Tohill *et al*., 2004). In particular, since the chemical induction method has not suggested an obvious *in vitro* neural differentiation, the undifferentiated autologous pSMSCs were used as transplant material for peripheral nerve regeneration in this study. Until now, except in this study, BMSCs (Azizi *et al*., 1998; Cuevas *et al*., 2004) and SKPs (Gorio *et al*., 2004; Marchesi *et al*., 2007) have been studied as undifferentiated multipotent cell sources for *in vivo* nerve regeneration. However, the regenerative mechanisms of transplanted cells have not been clearly understood. Recently, several researchers have proposed theories such as transdifferentiation, cell fusion and feeder theories (Yamaguchi *et al*., 2006).

In the present study, we have isolated pSMSCs, which had different characters to SKPs although their origins were similar, and observed histologically remarkable nerve regeneration, including incremental expression of S-100 and p75NGFR, after transplantation of autologous pSMSCs into peripheral nerve defects. S-100 protein is normally derived from neural crest cells, Schwann cells and glial cells; however, many other cells and inflammatory conditions are also related to this protein (Donato, 2003). The low-affinity nerve growth factor receptor, p75NGFR, was secreted during the early period of nerve injury and repair (Byun *et al*., 2008; Frostick *et al*., 1998). The high expression of these proteins at 4 weeks after cell transplantation suggests that active molecular nerve regeneration activities occur during that time period. In addition, pretransplanted labelled PKH26 was detected in the regenerated nerve tissues at 2 and 4 weeks post-transplantation, although their expression ratio was weakened as the cell graft time passed; this might have been related to the short half-life of PKH26. Taking of these data together, we could presume that *in vivo* transplanted autologous pSMSCs were well preserved and directly differentiated into nerve cells in the resected nerve gaps. In particular, distinguishable normally-structured nerve fibre regeneration, including axon, myelin sheath and peri- and epi-neurium, was observed in specimens 4 weeks after transplantation. However, in this specimen, the interstitial tissues between regenerated nerves were also highly constructed, resembling overgrown neoplasm-like tissues.

Although further studies are needed to evaluate the long-term effects of these overgrown tissues after *in vivo* cell transplantation, it is certain that autologous pSMSC transplantation showed better *in vivo* nerve regeneration potential than in the non-cell transplantation control group. Our results suggest that autologous SMSCs and fibrin glue scaffold may act as an available substitute for nerve conduit material in peripheral nerve defect sites.

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References

Azizi SA, Stokes D, Augelli BJ, *et al*. 1998; Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats-similarities to astrocyte grafts. *Proc Natl Acad Sci USA* **95:** 3908–3913.

Barnabé GF, Schwindt TT, Calcagnotto ME, *et al*. 2009; Chemically-induced RAT mesenchymal stem cells adopt molecular properties of neuronal-like cells but do not have basic neuronal functional properties. *PLoS One* **4**(4): e5222.

Blanpain C, Fuchs E. 2006; Epidermal stem cells of skin. *Annu Rev Cell Dev Biol* **22:** 339–373.

Byun JH, Lee JH, Choi YJ, *et al*. 2008; Co-expression of nerve growth factor and p75NGFR in the inferior alveolar nerve after mandibular distraction osteogenesis. *Int J Oral Maxillofac Surg* **37:** 467–472.

Bossolasco P, Cova I, Calzarossa C, *et al*. 2005; Neuroglial differentiation of human bone marrow stem cells *in vitro*. *Exp Neurol* **193:** 312–325.

Cuevas P, Carceller F, Garcia-Gomez I, *et al*. 2004; Bone marrow stromal cell im-

plantation for peripheral nerve repair. *Neurol Res* **26:** 230–232.
Deng W, Obrocka M, Fischer I, *et al*. 2001; *In vitro* differentiation of human marrow stromal cells into early progenitors of neural cells by conditions that increase cyclic AMP. *Biochem Biophys Res Commun* **282:** 148–152.
Dezawa M, Takahashi I, Esaki M, *et al*. 2001; Sciatic nerve regeneration in rats induced by transplantation of *in vivo* differentiated bone-marrow stromal cells. *Eur J Neurosci* **14:** 1771–1776.
Dezawa M. 2008; Systemic neuronal and muscle induction systems in bone marrow stromal cells: the potential for tissue reconstruction in neurodegenerative and muscle degenerative diseases. *Med Mol Morphol* **41:** 14–19.
Donato R. 2003; Intracellular and extracellular roles of S100 proteins. *Microsc Res Tech* **60:** 540–551.
Dyce PW, Zhu H, Craig J, *et al*. 2004; Stem cells with multilineage potential derived from porcine skin. *Biochem Biophys Res Commun* **316:** 651–658.
Frostick SP, Yin Q, Kemp GJ. 1998; Schwann cells, neurotrophic factors, and peripheral nerve regeneration. *Microsurgery* **18:** 397–405.
Gorio A, Torrente Y, Madaschi L, *et al*. 2004; Fate of autologous dermal stem cells transplanted into the spinal cord after traumatic injury (TSCI). *Neuroscience* **125:** 179–189.
Heine W, Conant K, Griffin JW, *et al*. 2004; Transplanted neural stem cells promote axonal regeneration through chronically denervated peripheral nerves. *Exp Neurol* **189:** 231–240.
Hou SY, Zhang HY, Quan DP, *et al*. 2006; Tissue-engineered peripheral nerve grafting by differentiated bone marrow stromal cells. *Neuroscience* **140:** 101–110.
Kang EJ, Byun JH, Choi YJ, *et al*. 2010; *In vitro* and *in vivo* osteogenesis of porcine skin-derived mesenchymal stem cell-like cells with a demineralized bone and fibrin scaffold. *Tissue Eng A* **16:** 815–827.
Kim BJ, Seo JH, Bubien JK, *et al*., 2002; Differentiation of adult bone marrow stem cells into neuroprogenitor cells *in vitro*. *NeuroReport* **13:** 1185–1188.
Lei Z, Yongda L, Jun M, *et al*., 2007; Culture and neural differentiation of rat bone marrow mesenchymal stem cells *in vitro*. *Cell Biol Int* **31:** 916–923.
Lu P, Blesch A, Tuszynski MH. 2004; Induction of bone marrow stromal cells to neurons: differentiation, transdifferentiation, or artifact? *J Neurosci Res* **77:** 174–191.
Lunney JK. 1994; Current status of the swine leukocyte antigen complex. *Vet Immunol Immunopathol* **43:** 19–28.
Marchesi C, Pluderi M, Colleoni F, *et al*. 2007; Skin-derived stem cells transplanted into resorbable guides provide functional nerve regeneration after sciatic nerve resection. *Glia* **55:** 425–438.
Mosahebi A, Woodward B, Wiberg M, *et al*. 2001; Retroviral labeling of Schwann cells: *In vitro* characterization and *in vivo* transplantation to improve peripheral nerve regeneration. *Glia* **34:** 8–17.
Munoz-Elias G, Woodbury D, Black IB. 2003; Marrow stromal cells, mitosis, and neuronal differentiation: stem cell and precursor functions. *Stem Cells* **21:** 437–448.
Murakami T, Fujimoto Y, Yasunaga Y, *et al*. 2003; Transplanted neuronal progenitor cells in a peripheral nerve gap promote nerve repair. *Brain Res* **974:** 17–24.
Neuhuber B, Gallo G, Howard L, *et al*. 2004; Reevaluation of *in vitro* differentiation protocols for bone marrow stromal cells: disruption of actin cytoskeleton induces rapid morphological changes and mimics neuronal phenotype. *J Neurosci Res* **77:** 192–204.
Ohyama M, Terunuma A, Tock CL, *et al*. 2006; Characterization and isolation of stem cell-enriched human hair follicle bulge cells. *J Clin Invest* **116:** 249–260.
Patel M, Mao L, Wu B, *et al*. 2007; GDNF-chitosan blended nerve guide: a functional study. *J Tissue Eng Regen Med* **1:** 360–367.
Pittenger MF, Mackay AM, Beck SC, *et al*. 1999; Multilineage potential of adult human mesenchymal stem cells. *Science* **284:** 143–147.
Riekstina U, Muceniece R, Cakstina I, *et al*. 2008; Characterization of human skin-derived mesenchymal stem cell proliferation rate in different growth conditions. *Cytotechnology* **58:** 153–162.
Rismanchi N, Floyd CL, Berman RF, *et al*., 2003; Cell death and long-term maintenance of neuron-like state after differentiation of rat marrow stromal cells: a comparison of protocols. *Brain Res* **991:** 46–55.
Rho GJ, Kumar BM, Balasubramanian S. 2009; Porcine mesenchymal stem cells – current technological status and future perspective. *Front Biosci* **14:** 3942–3961.
Sanchez-Ramos J, Song S, Cardozo-Pelaez F, *et al*. 2000; Adult bone marrow stromal cells differentiate into neural cells *in vitro*. *Exp Neurol* **164:** 247–256.
Shi C, Zhu Y, Su Y, *et al*. 2006; Stem cells and their applications in skin-cell therapy. *Trends Biotechnol* **24:** 48–52.
Shimizu S, Kitada M, Ishikawa H, *et al*. 2007; Peripheral nerve regeneration by the *in vitro* differentiated human stromal cells with Schwann cell property. *Biochem Biophys Res Commun* **359:** 915–920.
Tohill M, Mantovani C, Wiberg M, *et al*. 2004; Rat bone marrow mesenchymal stem cells express glial markers and stimulate nerve regeneration. *Neurosci Lett* **362:** 200–203.
Toma JG, McKenzie IA, Bagli D, *et al*. 2005; Isolation and characterization of multipotent skin-derived precursors from human skin. *Stem Cells* **23:** 727–737.
Vacanti V, Kong E, Suzuki G, *et al*. 2005; Phenotypic changes of adult porcine mesenchymal stem cells induced by prolonged passaging in culture. *J Cell Physiol* **205:** 194–201.
Wang FW, Jia DY, Du ZH, *et al*. 2009; Roles of activated astrocytes in bone marrow stromal cell proliferation and differentiation. *Neuroscince* **160:** 319–329.
Woodbury D, Reynolds K, Black IB. 2002; Adult bone marrow stromal stem cells express germline, ectodermal, endodermal, and mesodermal gene prior to neurogenesis. *J Neurosci Res* **96:** 908–917.
Woodbury D, Schwarz EJ, Prockop DJ, *et al*. 2000; Adult rat and human bone marrow stromal cells differentiate into neurons. *J Neurosci Res* **61:** 364–370.
Yamaguchi S, Kuroda S, Kobayashi H, *et al*. 2006; The effect of neuronal induction on gene expression profile in bone marrow stromal cells (BMSC) – a preliminary study using microarray analysis. *Brain Res* **1087:** 15–27.
Yoshii S, Ito S, Shima M, *et al*. 2009; Functional restoration of rabbit spinal cord using collagen-filament scaffold. *J Tissue Eng Regen Med* **3:** 19–25.

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