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Original Article

Neonatal mice muscle-derived stem cells differentiate into Schwann cell-like cells in vitro

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Abstract: Purpose: Muscle, as the most abundant organ, has been extensively used to study adult stem cells. In this study, we examined the biological characteristics of muscle-derived stem cells (MDSCs) and further evaluated the feasibility of inducing MDSCs under *in vitro* conditions to differentiate into cells exhibiting characteristics similar to those of Schwann cells (SC). Methods: We isolated multipotent muscle-derived stem cells (MDSCs) from neonatal skeletal muscle using a modified preplate technique. To characterize transdifferentiated MDSCs, we used immunocytochemistry to induce the expressions of select markers following a general observation of their morphology. Results: MDSCs exhibit grow patterns similar to that of stem spheres in stem cell medium, gaining the ability to self-renew and differentiate into the myotubes and myofibers. MDSCs express the hematopoietic stem cell marker (Sca-1) as well as a muscular cellular surface protein (Desmin). Immunocytochemical staining indicated that the treated cells were a subset of cells expressing the glial cell markers (S100β) and low-affinity nerve growth factor receptor ($P75^{NTR}$), indicative of differentiation towards SC phenotypes. Conclusion: Our *in vitro* study is a proof-of-concept for the viability of MDSC differentiation to exhibit SC-like characteristics. Further *in vitro* and *in vivo* studies are required, however, to clarify and address the observed limitations and to fully develop MDSCs therapy for peripheral nerve injury degeneration.

Keywords: Muscle-derived stem cells, Schwann cell, cell differentiation

Introduction

Peripheral nerve injuries are common occurrences and represent a major economic burden for society [1]. Clinical treatment conventionally involves surgical suturing of the damaged nerves for minor defects whereas for larger gaps, transplantation of a nerve graft is often necessary to facilitate nerve regeneration and functional recovery [2]. Despite innovative surgical advances such as microsurgery, functional recovery is often poor even with careful adherence to surgical procedures. Alternatives to conventional autografts have long been sought due to its associated drawbacks; the emergence of regenerative medicine and tissue engineering techniques, which enhance beneficial endogenous responses to nerve injury and promote the development of artificial nerve grafts, offers such potential alternatives [3].

Schwann cells (SCs) are the primary structural and functional cells that play a vital role in the process of peripheral nerve regeneration. When peripheral nerves are damaged, Wallerian degeneration occurs at the distal stump [4, 5]. The disruption of normal SC-axon interaction caused by nerve injury progressively results in the dedifferentiation of SCs and the activation of a growth promoting phenotype [3]. SCs removed degenerated axons and myelin debris and proliferate within the distal nerve segment to form "Bands of Büngner" to direct regenerating axons across the lesion, which is indispensable for nerve function reconstruction

[6]. Meanwhile, SCs synthesize and secrete a variety of neurotrophic factors, cytokines and cell adhesion molecules that guide and promote axons regeneration, and establish favorable microenvironments for constructing precise innervation [7-9]. It was an accomplishment to find that after seeding in artificial nerve conduits, SCs behave in a manner that enhances nerve regeneration [10-12]. SCs have thus become the most important and commonly-used seeds for developing "tissue engineered nerves". Autogenic SCs, however, can evoke additional morbidity and are difficult to obtain in large numbers. Furthermore, allogeneic SCs are involved in immunological rejections. Instead, ideal transplantable cells should be easily accessible, and could proliferate rapidly in culture and successfully integrate into host tissue with immunological tolerance [13]. Therefore, considerable efforts have been focused on inducing other cells to turn into SCs in order to expand alternatives sources.

Muscle-derived stem cells (MDSCs) are considered to be distinct from satellite cells as they may not be restricted to the myogenic lineage or mesenchymal tissues, and can differentiate into multiple lineages [14-17], while satellite cells have long been considered mono-potential stem cells capable of giving rise only to cells of the myogenic lineage. Controversy regarding the relationship between MDSCs and satellite cells persisted until Pax7 was investigated, but the origin of MDSCs remains to be unknown, and their relationship is also unclear [18], with the former potentially representing a predecessor of the latter [19]. Researchers have demonstrated that MDSCs obtained from later preplate populations displayed a superior ability to regenerate cells affected by Duchenne muscular dystrophy (DMD) [20, 21]. MDSCs have also been used in therapy for urinary incontinence and neurogenic bladder, as well as in bladder reconstruction after cystectomies [22-26]. Studies have also demonstrated that MDSCs possess a great potential for a variety of tissue engineering applications including the regeneration and repair of skeletal and cardiac muscle, bone, articular cartilage and peripheral nerves [27-37], positioning MDSCs as a promising candidate for future clinical cell-therapy endeavors. Besides the capability of renewal and multi-lineage differentiation into other stem cells, MDSCs have also been reported to exhibit long-term proliferation and immune-privileged behavior in both in vitro and in vivo conditions [38].

In this study, we examined the biological characteristics of muscle-derived stem cells (MDSCs) and further evaluated the feasibility of inducing MDSCs under *in vitro* conditions to differentiate into cells exhibiting characteristics similar to those of Schwann cells (SC). To characterize transdifferentiated MDSCs, we used immunocytochemistry to induce the expressions of select markers following a general observation of their morphology.

Materials and methods

Animals and experimental design

All experiments, including MDSCs isolation and SC-conditioned medium collection were performed on normal neonatal C57BL/6 (3-5 d) mice weighing 5-10 g. The mice were offered by the Second Military Medical University Experimental Animal Center [production license for SCXK (shanghai 2007-0003), animal use license for SYXK (shanghai 2007-0002)]. Their use was in compliance with the international guide principles for animal research and was approved by the animal care committee at Second Military Medical University.

MDSCs isolation, purification and immunohistochemistry

Primary muscle-derived cells were isolated and purified from the hind-limb muscles of 3-5 days C57BL/6 via a previously described modified preplate technique [15, 39]. In brief, the hind-limb muscles were collected under microscopy with unwanted tissues removed, including nerves, fat and fascia. The muscle was then washed by PBS 3 times and minced into a coarse slurry using scalpels. The muscle tissue was digested sequentially at 37°C in 0.2% collagenase-type II (Sigma-Aldrich) for 1 hour and then centrifuged at 1,000 rpm for 5 min. The cells were collected, incubated in dispase, prepared as 2.4 units/ml PBS for 45 min, and incubated for 30 min in 0.25% trypsin-EDTA diluted in PBS. After enzymatic dissociation, the released muscle cells were centrifuged and resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with a 10% horse serum (HS), 10% fetal bovine serum

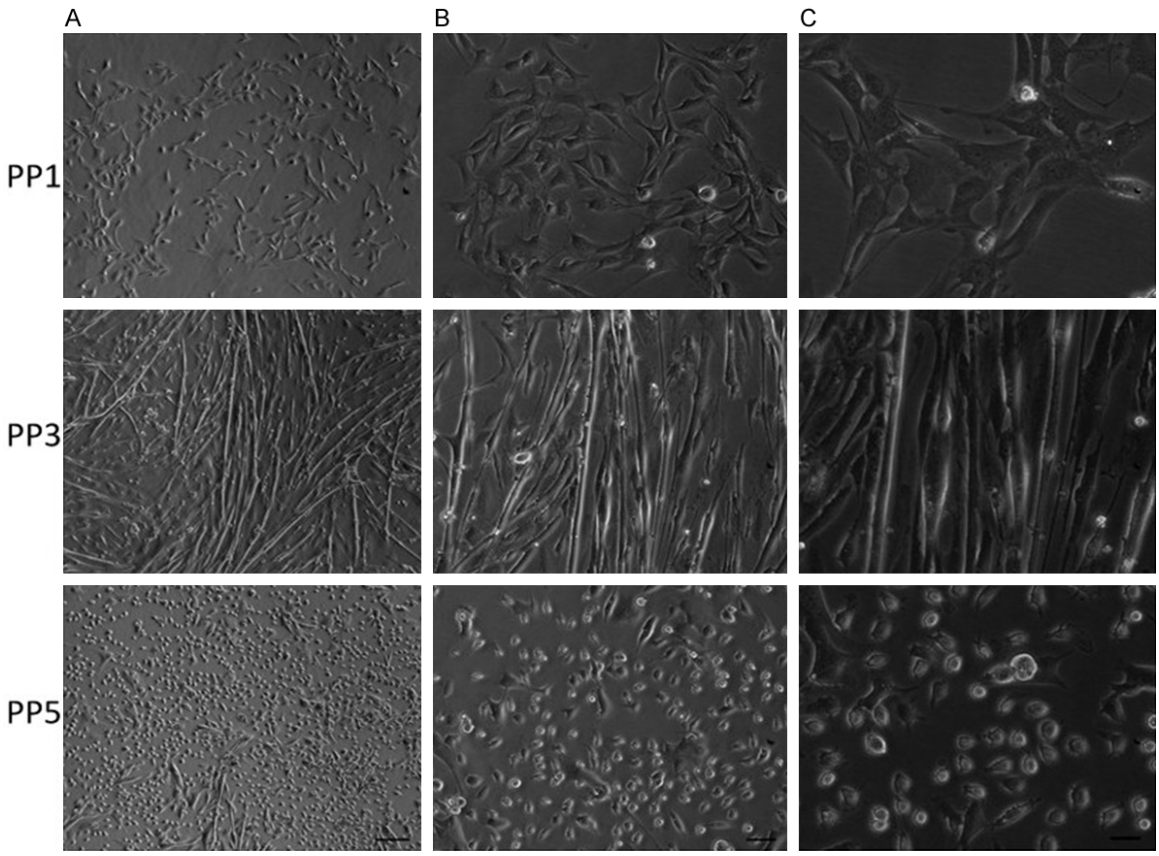

Figure 1. Morphology of LP cells at different stages. PP1 Appearance of rapidly adhering cells (RAC). RACs appear flat with fibroblast-like shape. PP3 Appear short spindle-shaped myogenic cells and myotubes. PP5 Slowly adhering cells (SACs) are mainly spherical and refractive. (bar A = 50 μm; bar B = 20 μm; bar C = 10 μm).

(FBS), 0.5% chick embryo extract mixture. The cell suspension was then plated in collagen type I-coated cell culture dishes. After the cells were incubated at 37°C in a humidified, 5% CO_2 incubator for two hours, early adhering cells attached to the dish; this cell population was labeled PP1 (preplate 1). The supernatant was withdrawn from the cell culture dishes and replated in fresh collagen-coated cell culture dishes, labeled as PP2. Fresh medium was added to the first set of adherent cells and after 24 hours, when 30%-40% of the cells had adhered to each cell culture dish, serial replating of the supernatant was repeated until PP6. The last cell suspension in the cell culture dish was maintained for 72 hours. To identify the MDSCs isolated from hind-limb muscle, they were fixed with a 4% paraformaldehyde solution for 30 minutes at 4°C and blocked with 5% goat serum at room temperature for 10 minutes. The cells were incubated overnight at room temperature in a humid chamber with the hematopoietic stem cell marker Sca-1 and the myogenic marker Desmin by immunofluorescence staining using rat anti mouse polyclonal anti-Sca-1 antibodies (1:100; Sigma) and rabbit anti mouse polyclonal anti-Desmin antibodies (1:80; Cell Signaling) as described [40]. After three rinses in PBS, they were incubated with Cy3 immunofluorescence-conjugated anti-rat IgG antibodies (1:500; Beyotime), fluoroisothiocyanate (FITC) immunofluorescence-conjugated anti-goat IgG antibodies (1:500; Beyotime) for 30 minutes at room temperature and Hoechst 33342 (1:1000; Invitrogen) for 5 minutes.

Schwann cell (SCs) culture, immunohistochemistry and conditional medium preparation

To culture SCs, sciatic nerves of neonatal mice (1 d) were dissociated bilaterally adhering to the method described by Brockes et al. [41] albeit with several modifications, and stored in Hank's balanced salt solution (HBSS, Sigma) with Ca^{2+} (-) and Mg^{2+} (-) at 0°C. The epineurium

and connective tissue were stripped from the cells with fine forceps under a microscope, and the sciatic nerves were minced into 2-3 mm fragments with fine scissors and dissociated with 0.25% trypsin at 37°C for 30 minutes. After incubation, the cell mixture was centrifuged at 103 rpm for 5 minutes, removing the supernatant. The cells were resuspended in DMEM/F12 (1:1) medium containing 10% FBS, 14 μM forskolin, 10 ng/ml basic fibroblast growth factor (bFGF; Invitrogen), and were placed in 35 mm Petri dishes pre-coated with poly-L-lysine (PLL, 10 mg/ml; Sigma). The cells were then incubated at 37°C with 5% CO_2. The culture medium was changed every 48 hours.

After four days in the primary culture, the SCs were fixed with 4% paraformaldehyde for 30 min at 4°C to identify those isolated from the murine sciatic nerve. After rinsing with PBS, primary antibodies were applied for 24 hours at 4°C using the following antibodies: rabbit anti mouse anti-S100β (1:100; Epitomics) and mouse anti-$P75^{NTR}$ (1:3200; Cell Signaling). After rinsing with PBS, the following secondary antibodies were applied for 90 minutes at room temperature: goat anti-mouse FITC-conjugated (1:500; Beyotime) and Cy3-red-conjugated IgG (1:500; Beyotime). Hoechst 33342 (1:1000; Invitrogen) was applied for 5 minutes. The staining was examined using a fluorescence microscope (Olympus).

When cell density reached 60% confluence, the culture medium was harvested as conditioned medium until 90% confluence. They were filtrated by a 0.22 μm sieve to get rid of impurities and cells.

Differentiation to a Schwann cell phenotype and immunocytochemistry

The stem cell growth medium was removed from sub-confluent MDSCs cultures at passage 2 and replaced with medium conditioned by SCs following a 3-cycle PBS washing. Four out of the 12-well culture plates were not changed with conditioned medium and remained as controls. Cells were washed and medium replaced with fresh conditioned medium every 48 hours.

To identify whether the treated MDSCs gave evidence to SCs phenotype, they were trypsinised and re-cultured on coverslip for immunostaining using the method outlined above. Cells were then incubated with rabbit anti mouse anti-S100β (1:100; Epitomics) and mouse anti-$P75^{NTR}$ (1:3200; Cell Signaling) respectively overnight at 4°C. After rinsing with PBS, the cells were incubated at room temperature with the following secondary antibodies: goat anti-mouse FITC-conjugated (1:500; Beyotime), Cy3-red-conjugated IgG (1:500; Beyotime) for 90 minutes and Hoechst 33342 (1:1000; Invitrogen) for 5 minutes. Staining was examined using an inverted fluorescence microscope (Olympus). All cell configurations were investigated under a phase-contrast microscope, and the immunoreaction above was visualized under a fluorescence microscope.

Results

Isolation and identification of MDSCs

MDSCS were found to be a subpopulation of muscle-derived cells isolated from the hindlimb muscles by the preplate method following different durations of time. Early preplate cultures (PP1-PP2) contained mostly fibroblasts, middle cultures (PP3-PP4) were mainly short spindle-shaped myogenic cells which could fuse into myotubes, whereas late cultures (PP5-PP6) were highly purified muscle-derived cells (**Figure 1**). In this study, these late preplate cells, LP cells, especially PP5, showed transition-amplifying cells (Figure S1A). The transition-amplifying cells expanded their volumes with the proliferation of the containing cells and released a large number of MDSCs upon disintegration. (Figure S1B, S1C). Trypsinized cells were obtained from PP6 after two weeks, the subculture of PP6 cells, had proliferating colonies of spherical, refractive, adherent cells (**Figure 2A-C**). Consistent with the previous reports [42], immunocytochemistry staining showed that these late preplate cells could express the hematopoietic stem cell marker Sca-1 (red: **Figure 2D**) and a muscular cellular surface protein Desmin (green: **Figure 2D**), indicating that these were MDSCs, and they were used for further experiments. Regarding cell growth, the initial cell density was 1×10^4/35 mm dish; generally, the cell number increased to 3×10^5 after five days, and calculations showed that the MDSC's doubling time was approximately 24 hours, slightly faster than the known growth rate of stem cells.

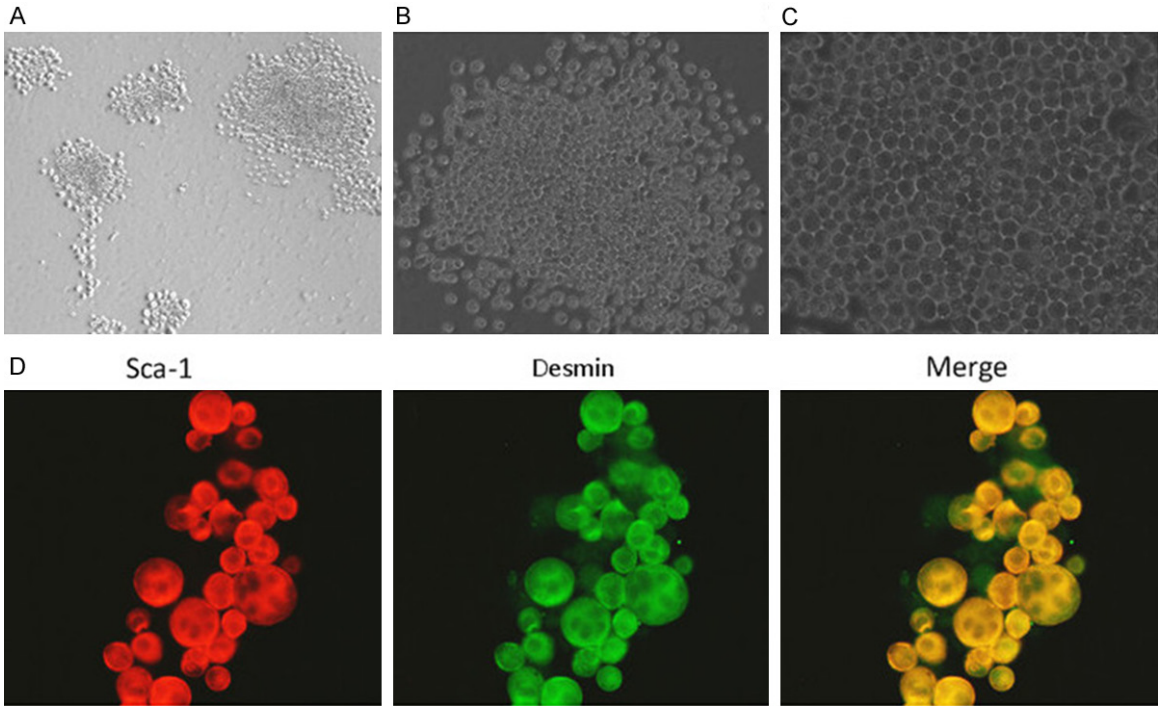

Figure 2. Morphology and identification of subcultured MDSCs. The LP cells form colonies and began to proliferate when passed the PP6 phase, appear to be small, round, refractive and sparse (A-C). Immunocytochemistry staining showed that these cells were Desmin$^+$ (green: D) and Sca-1$^+$ (red: D), identifying them to be MDSCs. *Blue* indicates Hoechst 33258$^+$. (bar A = 50 μm; bar B, D = 20 μm; bar C = 10 μm).

Characteristics of SCs

SCs were observed after two hours of suspension in the primary culture. The cells grew into a 40% confluence in 72 hours and a 90% confluence in a week with its distinct morphology (**Figure 3A-C**). Immunocytochemistry staining showed that these SCs were positive for S100β (green: **Figure 3D**) and P75^{NTR} (red: **Figure 3D**).

MDSCs can be induced into SC-like cells

The original cultured MDSCs were digested and passaged before the conditioned medium was induced (Before: **Figure 4A-C**). When the cell density reached about 80% confluence, the culture medium was added. After a one-week culture, spherical MDSCs began to stretch and halation was no longer present, leaving short spindle-shaped cell bodies with two axon process-like extensions that were cross-linked to form networks (After: **Figure 4D**). On the other hand, the processes of other MDSCs were stunted, causing the cells to float until death.

To confirm that the MDSCs had committed to the SCs phenotype, we performed immunocytochemistry after induction. After one week of induction, expression of S100β protein was exhibited evenly across the cytoplasms of MDSCs. The protein was concentrated around the nucleus. Most of S100β positive cells displayed the spindle-shaped appearance, while a few of them were large and flattened in shape (green: **Figure 4D**). Similarly, most of MDSCs appeared P75^{NTR}-positive (red: **Figure 4D**). The nuclei of transdifferentiated cells from MDSCs were stained by Hoechest (blue: **Figure 4D**) and were counted as an aggregate value. In the same area, the cells positive for S100β and p75^{NTR} were counted as well. Two merged micrographs showed a clearer understanding of the phenomenon (Merge: **Figure 4D**). A positive rate of 65.1% for S100β and 72.3% for P75^{NTR} was achieved.

Discussion

Tissue reconstructive engineering with multipotent stem cells is the imminent trend for the clinical treatment. The concept of tissue engineering, emerging in 1989, has received enthusiasm from regenerative medicine and plastic surgery communities. Research efforts were

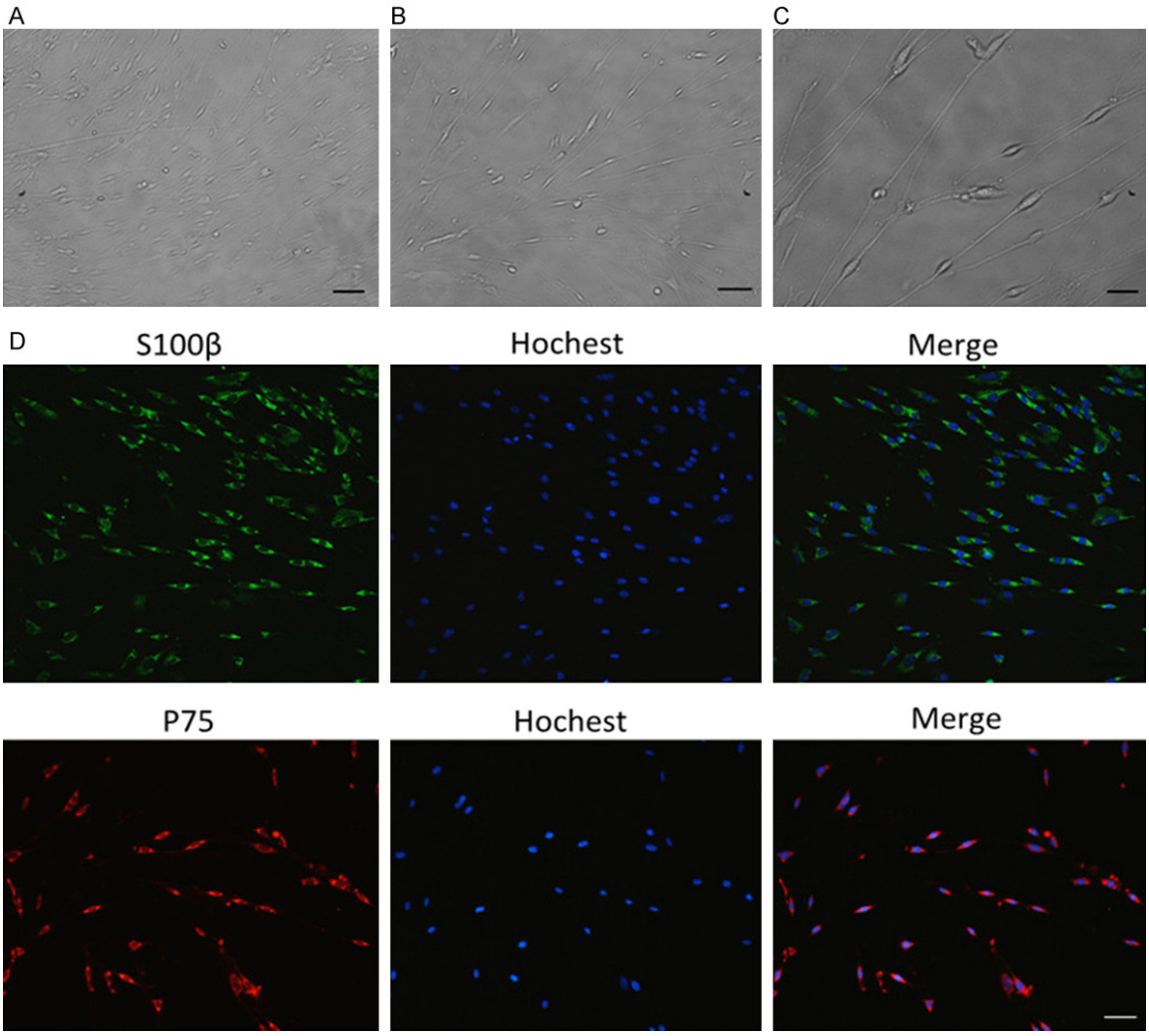

Figure 3. Morphology and identification of SCs. Mouse Schwann cells reached a confluence of 90% in a week after primary culture, with its morphology as fusiform (A-C). Immunocytochemistry staining showed that they were S100β^{+} (green: D) and P75^{NTR+} (red: D). *Blue* indicates Hoechst 33258^{+}. (bar A, D = 50 μm; bar B, D = 20 μm; bar C = 10 μm).

made to investigate various potential seeding cells - the core component of tissue engineering. In current stem cells and regenerative medicine, researchers have concentrated and have ultimately succeeded in isolating adult stem cells for research work and therapeutic use from all such kinds of tissue as bone marrow [43], adipose [44], and blood [45], to avoid the ethical issues within embryo stem cell research [46, 47]. These stem cells have the ability to differentiate into target tissues following the addition of different growth factors, cytokines, and culture medium stimuli. Skeletal cells, chondrocytes, adipocytes, and muscle cells are commonly used. Muscle tissue is a potential, viable source of adult stem cells due to its abundance and the low morbidity of the harvesting site. The rationale of isolating MDSCs as seeding cells from neonatal mice skeletal muscle for tissue engineering research is advocated by the article.

In this study, we plotted the growth curve of MDSCs by the way of cell counting under the microscope. We found that the growth curve was the S-type (data not shown). Cell proliferation has undergone three phases: adjustment period, logarithmic growth phase, and stable phase. When the number of cells reached a certain level, cell growth slowed down and began to enter the stable phase. The initial analysis has shown that the doubling time for

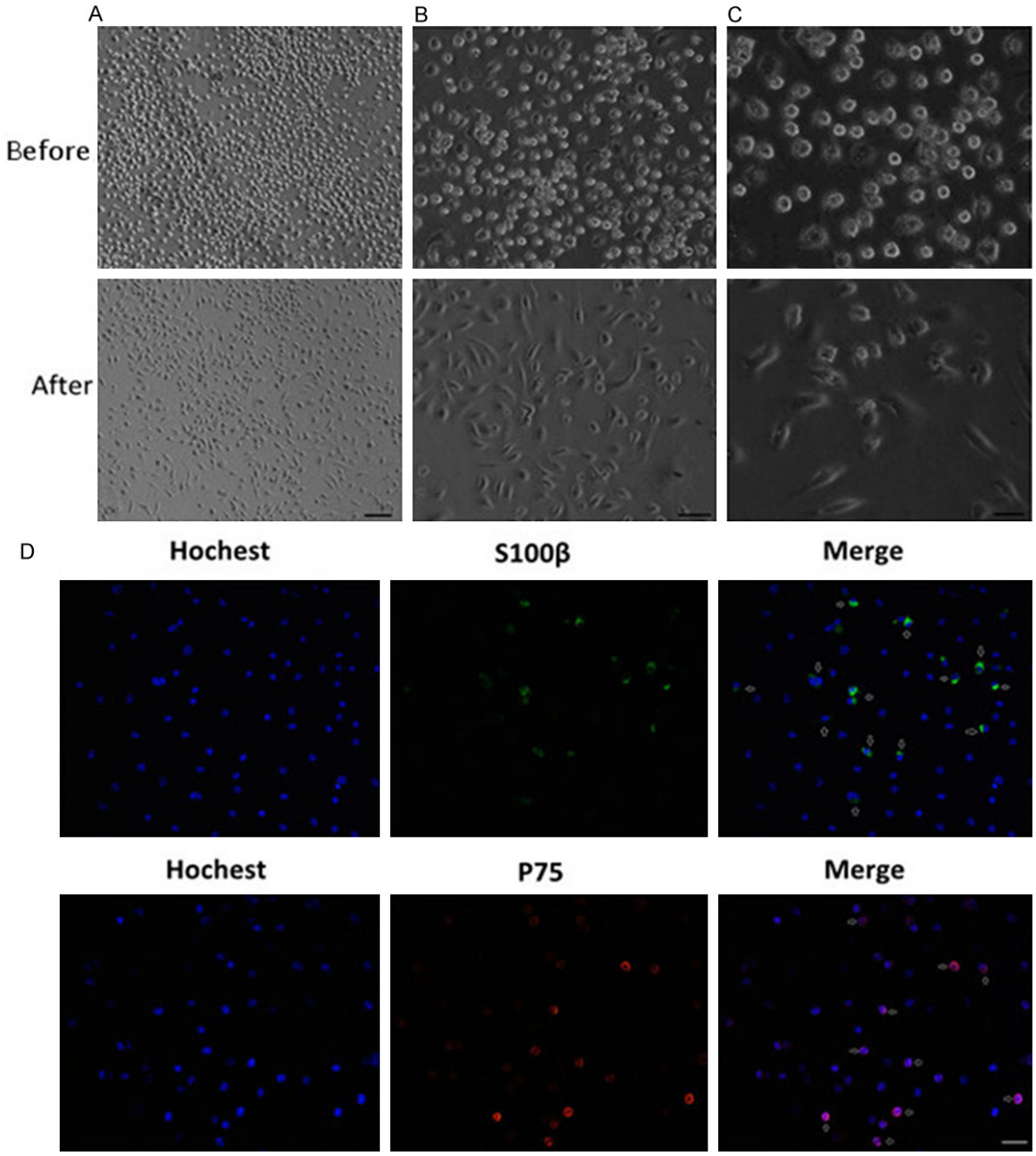

Figure 4. Morphology of MDSCs before and after induction and identification of MDSCs as SC-like cells. MDSCs appear to be small, spherical, refractive and adherent cells before induction (Before: A-C). After induction with SCs conditional medium, MDSCs began to stretch and the halation disappeared, leaving a short spindle-shaped cell body with two processes (After: A-C). In immunocytochemistry staining, the induced MDSCs showed an intense staining for S100β (green: D) and $P75^{NTR}$ (red: D). *Blue* indicates Hoechst 33258^{+}. (bar A = 50 μm; bar B, D = 20 μm; bar C = 10 μm).

MDSCs is 24 hours. The number of MDSCs reached the highest proliferation after 2 weeks of subcultivation. In other words, with regards to either cell number or cell proliferation, the subculture of MDSCs proved to be suitable for *in vitro* differentiation toward SC-like cells, which were used as seed cells for constructing "tissue engineered nerves". Although specific cell surface markers have not yet been isolated to definitively identify MDSCs that could express muscle, epithelial, mesenchymal and endothelial cell surface markers, it is generally considered that MDSCs are positive for Sca-1, CD34, Bcl-2, Desmin, CD56, etc [15, 38, 48]. In

the present study, two of the foregoing surface markers, Sca-1 and Desmin, were selected for characterizing the cultured MDSCs. The results demonstrated that MDSCs were positive for Sca-1 and Desmin (the hematopoietic stem cell marker and muscular cellular surface protein, respectively). The data indicates that MDSCs are a potential autologous cell source, able to aide tissue repair, or regeneration of damaged or diseased musculoskeletal tissues, and to assist in tissue engineering [49, 50].

Previous studies have indicated that MDSCs are a class of multipotent stem cells that have the capability of transdifferentiating into ectoderm-derived nerve tissue cells. In this study, the conditioned medium was adopted for inducing MDSCs to differentiate towards SC-like cells, as well as changes in morphological and immunohistochemical phenotypic characteristics of MDSCs towards nerve tissue cell lineage. Identification of the exact mechanisms will be a subject for future investigation. The transdifferentiation from MDSCs towards the SC phenotype is considered meaningful. It has been proven that SCs are able to produce neurotrophic factors that activate specific cell surface receptors, which initiate a cascade of intracellular events modifying neuronal morphology, survival, and functional capacity, and represent prompting efficiency in the neuronal regeneration [51]. Except for the ability to self-renew and the potential for multi-lineage differentiation, MDSCs have an immunological privilege, which would facilitate cell transplantation [38]. In this study, the MDSCs were co-cultured with SC-conditioned medium and were then stained positive for S100β and $P75^{NTR}$, indicating the primary transdifferentiation method from MDSCs towards the SC phenotype.

Our *in vitro* study is a proof-of-concept for the possibility of MDSCs differentiating into SC-like cells. There exist, however, several limitations within this study. Our study was conducted in *in vitro* conditions, and studies *in vivo* for cell viability and regenerative potential of MDSCs are necessary to determine whether SC-like cells are truly ideal for peripheral nerve injury therapy. Moreover, the factor that plays the pivotal role in the differentiation procedure of MDSCs must be isolated. The mixture conditioned medium produced by SCs included multi-neurotrophic and nerve growth factors confounded the source of transdifferentiation. Furthermore, SC-oriented transdifferentiation method using GGF/PDGF and other consecutive factors, which has been proved successful within ADSC [52] and MSC [53], did not yield the expected results. The transdifferentiation from neonatal mice-skeletal-muscle-derived stem cells to cells with SC phenotypes has provided another candidate for an alternative for SC. It is clear that the unrivaled potential of this therapeutic approach combined with the immediate need to answer relevant questions underlines the need for further investigation and continuing basic research to elucidate the underlying mechanisms of this stem cell differentiation. Therefore, further in vitro and in vivo studies are required to clarify and address the observed limitations, and to fully develop MDSCs therapy for peripheral nerve injury degeneration.

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Disclosure of conflict of interest

None.

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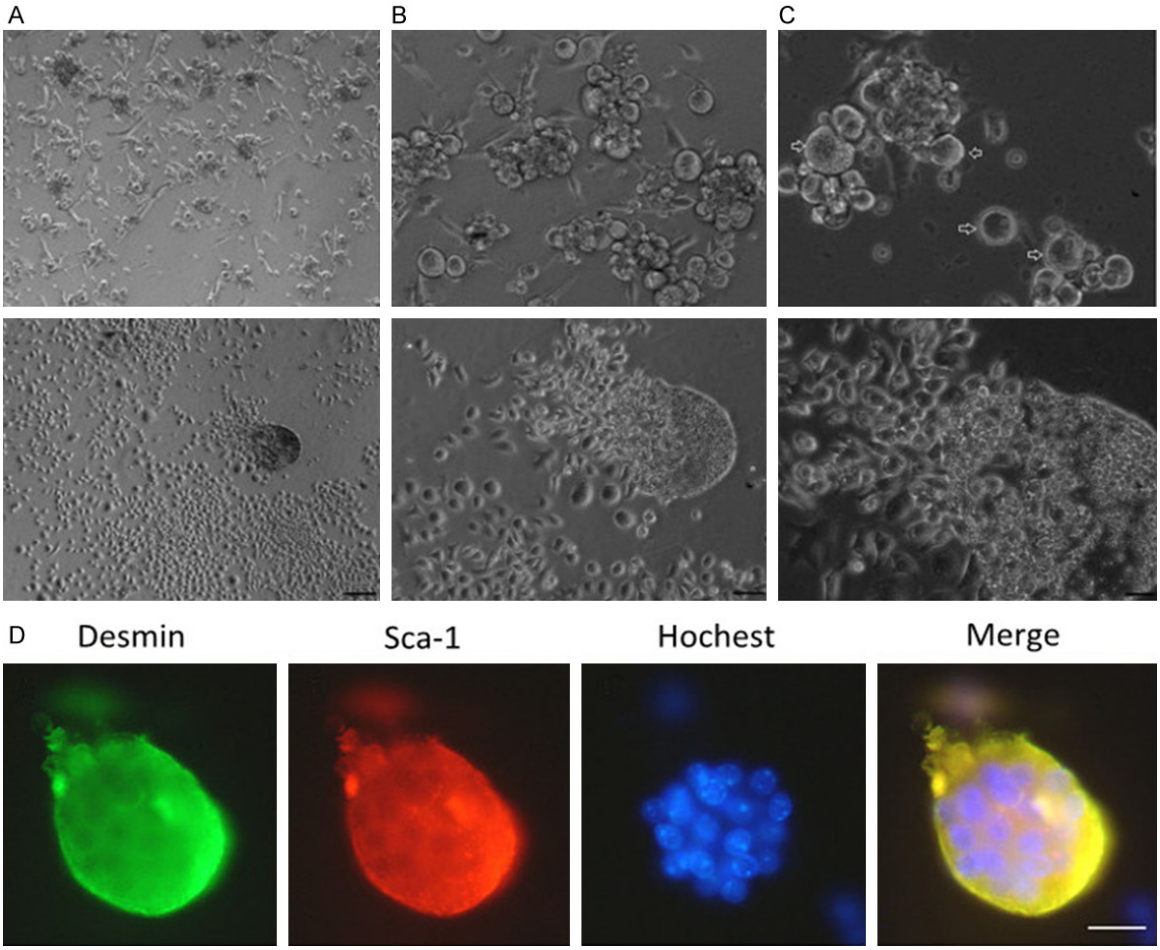

Figure S1. Morphological and biochemical characteristics of PP5 cells. The obvious morphological difference of PP5 phase was that the advent of transition amplifying cells and disintegration cells. (A-C) In immunocytochemistry staining, the PP5 cells also showed the stem cell properties by expressing Desmin (green: D) and Sca-1 (red: D), (bar A = 50 μm; bar B, D = 20 μm; bar C = 10 μm).