PDGF, NT-3 and IGF-2 in Combination Induced Transdifferentiation of Muscle-Derived Stem Cells into Schwann Cell-Like Cells

Yi Tang1,2,3, Hua He4,5, Ning Cheng4,5, Yanling Song1, Weijin Ding1, Yingfan Zhang1, Wenhao Zhang5, Jie Zhang1, Heng Peng6, Hua Jiang4*

1 Department of Plastic Surgery, Changzheng Hospital, Second Military Medical University, Shanghai, China, 2 Department of Plastic Surgery, No. 411 Hospital of CPLA, Shanghai, China, 3 Department of Neurosurgery, Changzheng Hospital, Second Military Medical University, Shanghai, China, 4 Department of Transfusion, Changhai Hospital, Second Military Medical University, Shanghai, China, 5 Department of Hematology, XinHua Hospital, Affiliated to Shanghai Jiao Tong University (SJTU) School of Medicine, Shanghai, China, 6 Department of Mathematics, Hong Kong Baptist University, Kowloon, Hong Kong

Abstract
Muscle-derived stem cells (MDSCs) are multipotent stem cells with a remarkable long-term self-renewal and regeneration capacity. Here, we show that postnatal MDSCs could be transdifferentiated into Schwann cell-like cells upon the combined treatment of three neurotrophic factors (PDGF, NT-3 and IGF-2). The transdifferentiation of MDSCs was initially induced by Schwann cell (SC) conditioned medium. MDSCs adopted a spindle-like morphology similar to SCs after the transdifferentiation. Immunocytochemistry and immunoblot showed clearly that the SC markers S100, GFAP and p75 were expressed highly only after the transdifferentiation. Flow cytometry assay showed that the portion of S100 expressed cells was more than 60 percent and over one fourth of the transdifferentiated cells expressed all the three SC markers, indicating an efficient transdifferentiation. We then tested neurotrophic factors in the conditioned medium and found it was PDGF, NT-3 and IGF-2 in combination that conducted the transdifferentiation. Our findings demonstrate that it is possible to use specific neurotrophic factors to transdifferentiate MDSCs into Schwann cell-like cells, which might be therapeutically useful for clinical applications.

Introduction
Schwann cells (SCs) play a crucial role in peripheral nerve development and regeneration, and are thus an attractive therapeutic target in peripheral nerve injuries [1–3]. It is reported that cultured SCs could induced neuronal sprouting and regrowth in cell culture experiments and improve peripheral nerve regeneration in vivo [4,5]. SCs can be obtained from nerve biopsies for autologous transplantation and will not elicit an intense immune response. However, it’s difficult to culture sufficient numbers of autologous SCs because of their restricted mitotic activity, and there are also other disadvantages such as limitations in the supply of nerve material [6,7]. Use of allogenic cells would need subsequent clinical immunosuppression [4]. Stem cells may be an alternative source for SCs. However, the clinical application of embryonic stem cells is limited because of ethical problems and their carcinogenic potential [8]. Increase evidence shows that adult stem cells may be promising candidate sources of cells [9,10].

Skeletal muscle may represent a convenient and valuable source of stem cells for stem cell-mediated gene therapy. Previous evidence supports the existence of MDSCs that exhibits both multipotentiality and self-renewal capabilities and therefore can be used for tissue engineering and regenerative therapy [11,12]. MDSCs have the ability to differentiate, upon stimulation with defined media, into multiple types of cells, including myogenic, hematopoietic, osteogenic, adipogenic, and chondrogenic-like cells [13]. The apparent advantages of MDSCs have led us to investigate whether they could be transdifferentiated to a Schwann cell phenotype.

Our aim was to assess the phenotypic and bioassay characteristics of MDSCs transdifferentiated to SC-like cells. Importantly, we also sought to determine the neurotrophic factors which directed the transdifferentiation.

Materials and Methods

Ethics Statement
All animal experiments were approved by the Administrative Committee of Experimental Animal Care and Use of Second Military Medical University (SMMU, Licence No. 2011023), and conformed to the National Institute of Health guidelines on the ethical use of animals.
Flow cytometry

Transdifferentiation of MDSCs to SC-like cells

Cultured MDSCs were stimulated with SC conditioned medium or medium containing different combinations of neurotrophic factors for three days. The morphological changes of the cells were studied using a light microscopy. Expression of SC markers S100, GFAP and p75 in the cells were analyzed by immunocytochemistry, flow cytometry and immunoblot.

Immunocytochemistry

The primary antibodies used in this study were rabbit anti-desmin (1:50, Cell Signaling), rat anti-Sca-1 (1:40, Sigma-Aldrich), mouse anti-S100 (1:40, Invitrogen). Cultured mouse MDSCs, SCs and tMDSCs were fixed and stained according to standard procedures [14].

Flow cytometry

The percentages of Sca-1 and desmin positive MDSCs were analyzed by flow cytometry. All antibodies used in this assay were from BioLegend. Live cell events were collected and analyzed on a FACSCalibur flow cytometer using Cell Quest software.

Immunoblot

Total proteins were extracted from cells (Cultured MDSCs, SCs, and tMDSCs) using sodium dodecyl sulfate lysis buffer. The protein were electrophoresed on SDS/PAGE and transferred to polyvinylidene fluoride membranes. The membranes were incubated with the primary antibodies followed by the horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies. The protein bands were visualized using the ECL system and scanned.

Enzyme-linked immunosorbent assay (ELISA)

The quantities of neurotrophic factors in the SC conditioned medium were measured using ELISA kit from Invitrogen. The tested medium was incubated in plates coated with capture antibodies (anti-BDNF, anti-PDGF, anti-NT-3, anti-IGF-2, anti-NGF, anti-GDNF, anti-FGF). After that, plates were incubated with secondary antibodies and then with peroxidase-conjugated anti-mouse IgG. Soluble colorimetric product was measured.

Statistical analysis

Statistical differences between two groups were determined by two-tailed Student’s t test. Multiple group comparisons were made by ANOVA test, using a significance level of 95%. Data were presented as means ± standard error of the mean.

Results

Transdifferentiation of MDSCs into SC-Like Cells

To obtain conditioned medium of SC which would be used for differentiation of MDSCs, we isolated SCs from mouse sciatic nerve and dorsal root ganglia. The isolated cells had typical spindle-shaped SC morphology (Fig. 2 A). The isolated cells were satellite cells adhered at approximately PP5 (Fig. S1). The cells of PP6 had a more rounded shape like stem cells (Fig. 1 A). Cell viability determined by trypan blue staining demonstrated that over 95% of the PP6 cells were viable (Data not shown). Cell growth assay showed that the cells of PP6 had a quiescent slow-cycling phenotype which was also a feature of stem cells (Fig. 1 B). To further demonstrate that the PP6 cells were MDSCs, we investigated the expression of Sca1 which was a well-defined marker for putative MDSCs in PP6 cells. Immunocytochemistry showed that the PP6 are Sca 1 positive and are also desmin positive which indicated they were muscle-derived (Fig. 1 C). Flow cytometry assay demonstrated that 93.23 ± 0.93% of the PP6 cells were Sca 1 positive, and 94.18 ± 0.38% were desmin positive. 90.1 ± 1.28% were double positive cells, indicating that most of the PP6 cells were MDSCs (Fig. 1 D). These results suggested that the PP6 cells isolated from mouse skeletal muscles were highly purified MDSCs.

Transdifferentiation of mouse MDSCs to a Schwann cell phenotype

To to obtain conditioned medium of SC which would be used for differentiation of MDSCs, we isolated SCs from mouse sciatic nerve and dorsal root ganglia. The isolated cells had typical spindle-shaped SC morphology (Fig. 2 A). The isolated cells were assessed for expression of the SC markers S100, GFAP, and p75. Immunocytochemistry with anti-S100 demonstrated they highly expressed S100 (Fig. 2 B). In addition, flow cytometry assay showed that most of the cells were S100 (96.77 ± 1.46%), GFAP (92.92 ± 4.94%), and p75 (93.38 ± 0.90%) positive, and 86.12 ± 1.53% of the cells expressed all of the three proteins (Fig. 2 C). Thus, the isolated cells were SCs with high purity.

Next, the conditioned medium of isolated mouse SCs was added to the cultured MDSCs to induce their differentiation. 72 hours after the induction, morphology of MDSCs was changed to...
spindle-like shape with processes, which was a typical morphology of SC-like cells (Fig. 3 A). The transdifferentiated SC-like cells (tMDSCs) were assessed for expression of the SC markers S100, GFAP, and p75 to study evidence of phenotypic progression to a SC lineage. Immunocytochemistry showed that the tMDSCs highly expressed the SC marker S100 protein (Fig. 3 B). Moreover, flow cytometry assay demonstrated that 65.48 ± 6.20%, 39.84 ± 1.66% and 41.08 ± 0.78% of the cells expressed all of the three proteins (Fig. 3 C). Immunoblot assay of the S100, GFAP and p75 expression showed an accord with flow cytometry (Fig. 3 D). The MDSCs expressed the SC markers highly only after transdifferentiation, indicating the tMDSCs were progressed along a SC lineage.

Neurotrophic factors essential for the transdifferentiation

It has been reported that neurotrophic factors secreted by SCs support the survival of neurons cultured in vitro and in vivo after peripheral nerve injury [16,17]. In this study, the conditioned medium of isolated mouse SC could induce the transdifferentiation of MDSCs to SC-like cells. We sought to determine the neurotrophic factors involved in this process.

First, we detected the quantity of seven neurotrophic factors in the conditioned medium, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), glial cell line-derived neurotrophic factor (GDNF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and insulin-like growth factor-2 (IGF-2). Quantitative method of ELISA was conducted and the results showed that there were high levels of BDNF, PDGF, NT-3 and IGF-2 in the conditioned medium (Fig. 4 A). The levels of NGF were relatively low, while GDNF and FGF were undetectable (Fig. 4 A). It seemed that...
BDNF, PDGF, NT-3, IGF-2 and NGF might be involved in the transdifferentiation of MDSCs to SC-like cells.

To determine which neurotrophic factors directed the transdifferentiation, we treated the MDSCs with one alone, two in combination or three in combination of the five neurotrophic factors and detected the morphological changes of the cells. None of the five neurotrophic factors could induce morphological changes of MDSCs alone, and the results were similar when MDSCs were treated with two factors in combination (data not shown). In the treatments of three neurotrophic factors in combination, only the combination of PDGF (1000 pg/ml), NT-3 (500 pg/ml) and IGF-2 (200 pg/ml) could induce the morphological change of MDSCs to SC-like cells (Fig. 4 B). Moreover, immunocytochemistry showed that PDGF, NT-3 and IGF-2 in combination also induced expression of SC marker S100 protein in MDSCs (Fig. 4 C). The results of flow cytometry were consistent with the SC conditioned medium induced transdifferentiation (Fig. 3 C), as the portion of S100, GFAP, and p75 positive cells were 58.64±4.38%, 47.38±0.84% and 44.33±2.39%, while 27.89±5.98% of the cells expressed all of the three proteins (Fig. 4 D). In addition, immunoblot assay demonstrated that treatment of PDGF, NT-3 and IGF-2 in combination could induce high expression of S100, GFAP, and p75 in transdifferentiated MDSCs (tMDSCs) (Fig. 4 E). These results indicated the effects of SC conditioned medium on MDSCs transdifferentiation might be mediated by PDGF, NT-3 and IGF-2 in combination.

**Discussion**

In this study, we showed that primarily cultured mouse MDSCs could be transdifferentiated to Schwann cell phenotype. The transdifferentiation could be induced by Schwann cell conditioned medium or by the combined treatment of three neurotrophic factors. Several kinds of evidence support the success of transdifferentiation. First, we observed that morphology of MDSCs was changed along a typical SC-like spindle-like shape with processes after the transdifferentiation. Second, we showed that the SC marker S100 was highly expressed in the tMDSCs using immunocytochemistry assay. Third, the immunoblot demonstrated clearly that the SC markers S100, GFAP and p75 were expressed highly only after the transdifferentiation. Finally, flow cytometry assay showed that the portion of S100 expressed cells was about 60 percent and over one fourth of the transdifferentiated cells expressed all the three SC markers, indicating an efficient transdifferentiation.

MDSCs are a potentially new type of undifferentiated cell isolated from skeletal muscle without myogenic restrictions. MDSCs have been reported to differentiate into different types of cells, including myogenic, hematopoietic, osteogenic, adipogenic, and chondrogenic-like cells [13]. MDSCs also have a remarkable long-term self-renewal and regeneration capacity [18]. Skeletal muscle is also a convenient source which could not induce the problem of clinically immunosuppression. These properties form the basis of potential clinical use of MDSCs in the therapies of degenerative diseases. In this study, we have successfully induced the transdifferentiation of MDSCs towards SC-like phenotype,
however, the true function of these SC like cells remains to be fully investigated.

Previous studies have shown that growth factors affect differentiation directly in stem cell populations [19]. However, there are not many reports about single factor which directs differentiation exclusively to one cell type. SCs developed through stages known as SC precursor cells, early SC and mature myelinating or non-myelinating SC. Several growth factors, such as bFGF, PDGF, neuregulin-1 (NRG-1) and its isoforms, neurotrophin-3 and IGF-1, have been reported involved in the development from SC precursor cells into early SC [20,21]. Adipose-derived stem cells treated with a mixture of glial growth factors (GGF-2, bFGF, PDGF, forskolin, PDGF-AA and Her-β) have proved to successfully induce the differentiation of bone marrow stromal cells to SC like cells [22]. Combinations of bFGF, PDGF-AA and Her-β have proved to successfully induce the differentiation of bone marrow stromal cells to SC like cells [23]. In this study, the combinations of PDGF, NT-3 and IGF-2 successfully induced transdifferentiation of MDSCs along SC like phenotype. From the neurotrophic factors detected in the Schwann cell conditioned medium, only this combination could induce the transdifferentiation. We also used the reported growth factors (GGF-2, bFGF, PDGF, forskolin, PDGF-AA and Her-β) for the transdifferentiation, but none of these growth factors or the reported combination had positive effects on MDSCs transdifferentiation. These results suggested that specific growth factors conduct the differentiation of different types of stem cells, even differentiation towards the same cell types.

Supporting Information

Figure S1 Morphology of cells in preplate method. (TIF)
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Author Contributions

Conceived and designed the experiments: HJ YT HH. Performed the experiments: YT HH NC YS. Analyzed the data: YT HH WZ HP. Contributed reagents/materials/analysis tools: WD YZ JZ. Wrote the paper: YT HH.

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Figure 4. Neurotrophic factors which direct the transdifferentiation. (A) ELISA assay showed the quantity of seven neurotrophic factors in the SC conditioned medium. There were high levels of BDNF, PDGF, NT-3 and IGF-2. The levels of NGF were relatively low, while GDNF and FGF were undetectable. (B) Phase-contrast micrographs showed that MDSCs adopted a SC-like shape upon treatment with PDGF (1000 pg/ml), NT-3 (500 pg/ml) and IGF-2 (200 pg/ml) in combination. (C) Immunocytochemistry showed that the tMDSCs highly expressed the SC marker S100 protein. (D) Flow cytometry assay demonstrated that the portion of S100, GFAP, and p75 positive cells in tMDSCs were 58.64 ± 4.38%, 47.38 ± 0.84% and 44.33 ± 2.39%, while 27.89 ± 5.98% of the tMDSCs expressed all of the three SC markers (data are mean % cells ± S.E.M.). (E) Immunoblot assay showed the MDSCs expressed of S100, GFAP and p75 only after transdifferentiation. β-actin served as loading control. Right, bar graph of qualitative data in statistics. S100, GFAP and p75 protein levels were normalized to that of β-actin, shown as mean ± S.E.M. **, p < 0.01 vs. MDSC.

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