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Protein hairy enhancer of split-1 expression during differentiation of muscle-derived stem cells into neuron-like cells★

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Abstract
Muscle-derived stem cells were isolated from the skeletal muscle of Sprague-Dawley neonatal rats aged 3 days old. Cells at passage 5 were incubated in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 20 μg/L nerve growth factor, 20 μg/L basic fibroblast growth factor and 1% (v/v) penicillin for 6 days. Cells presented with long processes, similar to nerve cells. Connections were formed between cell processes. Immunocytochemical staining with neuron specific enolase verified that cells differentiated into neuron-like cells. Immunofluorescence cytochemistry and western blot results revealed that the expression of protein hairy enhancer of split-1 was significantly reduced. These results indicate that low expression of protein hairy enhancer of split-1 participates in the differentiation of muscle-derived stem cells into neuron-like cells.

Key Words
muscle-derived stem cells; neuron-like cells; protein hairy enhancer of split-1; proliferation; neuron specific enolase; neural regeneration

Research Highlights
(1) Prolonged culture duration decreased the number and ability of rat muscle-derived stem cells to differentiate.
(2) The medium containing nerve growth factor and basic fibroblast growth factor can induce the differentiation of rat muscle-derived stem cells into neuron-like cells.
(3) Protein hairy enhancer of split-1 expression diminished during the differentiation of rat muscle-derived stem cells into neuron-like cells.
(4) Hairy enhancer of split-1 regulated the proliferation and differentiation of rat muscle-derived stem cells.
(5) Muscle-derived stem cell transplantation may be used for the treatment of spinal cord injury.

Abbreviations
Hes1, hairy enhancer of split-1; NSE, neuron specific enolase; MDSCs, muscle-derived stem cells

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INTRODUCTION

Spinal cord injury is a devastating and traumatic condition that predominantly affects young males, with an annual incidence of 15–40 cases per million[1]. Currently, there are no effective treatment methods. However, the emergence of tissue engineering has brought new hope to these diseases, especially the application of stem cell research, which has been the focus in recent years. By transplanting cells to injured areas, some regions have been restored[2]. Muscle-derived stem cells (MDSCs), which are different from satellite cells, are a type of adult stem cell. Moreover, MDSCs have lasting and a strong self-renewal capacity. They also have the ability to differentiate into different kinds of cells, including neural cells, bone cells, and smooth muscle cells[3-7] following specific stimulation *in vitro*. However, the mechanism of how these cells differentiate into specific cell types remains unclear. The Notch target hairy enhancer of split-1 (Hes1), a repressive transcription factor[8], is one of the main ligands for the Notch acceptor that exists in the mammalian cell membrane. The Hes family of basic helix loop helix (bHLH)[9] transcriptional repressor influences progenitor cell proliferation and differentiation. Hes1, which is expressed in marrow, fetal liver stromal-cells, thymic epithelial cells and other cell types, participates in the regulation of growth and development, and is a single transmembrane glycoprotein[10-11]. Hes1 is predominantly involved in the inhibition of precursor cell differentiation signals that occur in the nervous system, muscle formation, endocrine and exocrine cells, T cells thymus selection and other important physiological processes[12-13]. A previous study showed that the Hes1 gene could inhibit cell differentiation and promote proliferation[14]. Moreover, Hes1 was shown to play an essential role in contact inhibition of cell proliferation in $3T_3$-L1 cells by repressing E_2F-1 expression[15]. However, the role of Hes1 in the differentiation of MDSCs into neuron-like cells remains to be determined. Therefore, in this study, we aimed to identify the role of Hes1 in MDSC differentiation and proliferation.

RESULTS

Morphology and desmin immunocytochemical identification of MDSCs after subculture

MDSCs were observed to have an irregular shape after subculture. Cell proliferation and migration occurred when passage number and time in culture increased, and cells gradually became regular in shape. These cells easily fused and their growth had directional properties (Figure 1).

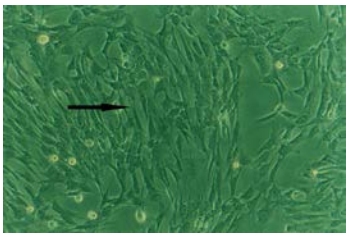

Figure 1 Muscle-derived stem cell cultures (inverted phase contrast microscope, × 100).

Passage 5 cells were bipolar and appeared spindle in shape (arrow). The nucleus had strong refraction and gradually grew to a regular form.

MDSCs were identified using the desmin primary antibody. Desmin-positive staining in the cytoplasm of passage 5 (P5) cells was observed. Five hundred cells were randomly observed, and 90% of these cells were found to be Desmin-positive. However, only a few positive cells were observed in P1 (Figure 2).

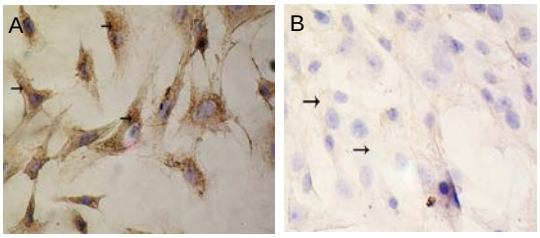

Figure 2 Desmin expression in muscle-derived stem cells (immunocytochemical staining, inverted phase contrast microscope).

(A) The cytoplasm of passage 5 (P5) cells was positive for desmin, and the ratio of positive cells was high. The arrows indicate desmin-positive (brown) staining in the cytoplasm, and the nucleus was stained blue (× 200).

(B) The cytoplasm of P1 cells was negative for desmin. The arrows show that the cytoplasm was negative for desmin, and the nucleus was stained blue (× 400).

Morphology of MDSCs after differentiation

After the cells were induced for 6 days in the experimental group, cell proliferation occurred and long processes, similar to those of nerve cells, were observed. Cell processes were also connected to each other. These changes took place gradually and the differentiation rate was high. Cell proliferation was not obvious and the formation of neurospheres was not detected (Figure 3), but the morphology of control cells did not change significantly.

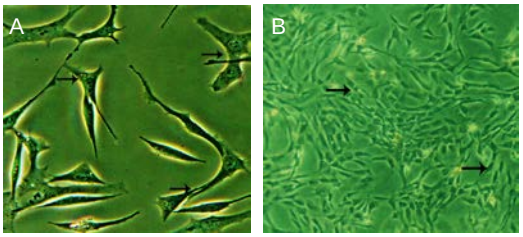

Figure 3 Morphology of muscle-derived stem cells after differentiation (inverted phase contrast microscope, × 100).

(A) The induced cells possessed long processes, and they seemed to be neuron-like cells. Cells (arrows) showed some contact between long processes and there were no neurospheres present.

(B) The cell morphology of the control group had no significant changes; spindle-shaped cells (arrows) appeared, without protrusions.

Neuron specific enolase (NSE) expression in MDSCs

After being induced for 6 days, the cytoplasm and processes of neuron-like cells stained by NSE exhibited strong immunoreactivity, whereas non-induced cells were negative for NSE (Figure 4).

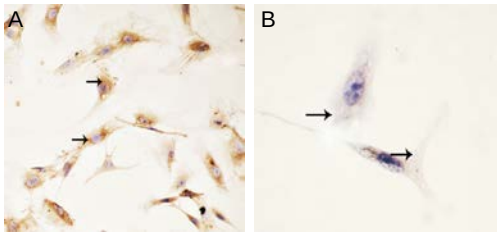

Figure 4 Expression of neuron specific enolase (NSE) after differentiation between the experimental group and the control group (immunocytochemical staining, inverted phase contrast microscope).

(A) The cytoplasm of neuron-like cells was positive for NSE (arrows), which was located in cell bodies and processes, and the ratio of positive cells was high at 6 days after induction in the experimental group (× 200).

(B) The cytoplasm of cells in the control group (arrows) was negative for NSE (× 400).

Hes1 protein expression in the two groups

Immunofluorescence cytochemistry staining results demonstrated that at 6 days after induction, Hes1 protein expression was minimal in the cytoplasm and processes of differentiated neuron-like cells when compared with non-induced cells (Figure 5).

Western blot assay showed that after being induced for 6 days, Hes1 protein expression was significantly reduced in the experimental group when compared with the control group ($P < 0.05$; Figure 6).

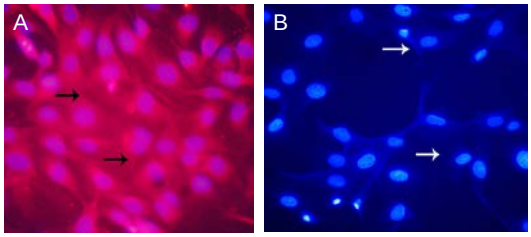

Figure 5 Expression of hairy enhancer of split-1 (Hes1) protein (specific staining of muscle-derived stem cells) after differentiation in the experimental group and the control group (immunofluorescence cytochemistry staining, fluorescence microscopy, × 400).

(A) In the control group, the red fluorescence (Hes1) was strong (arrows), and nuclei stained blue.

(B) The cytoplasm of neuron-like cells was negative for Hes1.There was no Hes1 present in the experimental group and blue staining was very strong in the nucleus (arrows).

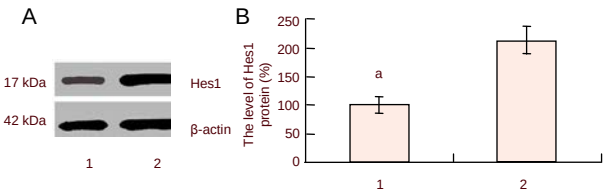

Figure 6 Hes1 protein expression as detected by western blot assay during the differentiation of muscle-derived stem cells into neurons.

(A) Western blot analysis showed that Hes1 was down-regulated in neuron-like cells induced after 6 days in culture.

(B) Western blot semi-quantitative analysis results. The data (absorbance ratio to β-actin) are expressed as mean ± SD; the experiment was repeated five times. $^{a}P < 0.05$, *vs.* control group using two samples *t*-test.

1: Experimental group; 2: control group.

DISCUSSION

There is a traditional viewpoint that injured axons in the central nervous system cannot be regenerated because of the poor intrinsic regeneration capacity and the inhibition of the external microenvironment[16]. However, transplantation of activated cells with tissue engineering methods can promote the repair and regeneration of injured axons and recover partial function of the spinal cord[17].

MDSCs are abundant and can be isolated, cultured and amplified *in vitro*[18]. The survival rate of MDSCs is significantly higher than that of satellite cells after transplantation[19]. Moreover, the ability to regulate an allogeneic immune response is easier, and these cells

may provide an autologous stem cell treatment for genetic diseases, degenerative diseases and the repair of injured tissues or organs. MDSCs have their own characteristic shape, and their myogenic properties can be identified through desmin staining. In addition, MDSCs have a multi-directional differentiation capacity. In this study, we hypothesized that the proliferation rate of MDSCs would gradually decline with the extension of cultivation time after birth in rats. However, if animals were too young, the proliferation rate of MDSCs was low. Newborn rats aged 1–3 days old were the most appropriate age for the isolation of MDSCs.

In this study, the combination of nerve growth factor and basic fibroblast growth factor in the differentiation medium induced MDSCs to differentiate into neuron-like cells *in vitro*. This result allowed us to proceed with our MDSC transplantation studies for the treatment of spinal cord injury.

The Notch gene was first discovered in Drosophila melanogaster, and embryonic development in Drosophila can be caused by mutations leading to over-differentiation of the nervous system[20]. Through molecular cloning techniques, it was confirmed that the Notch gene in Drosophila encoded for one kind of membrane protein receptor[21]. The Notch signaling pathway is involved throughout animal development and is a major regulator of cell proliferation and differentiation[22]. In addition, it plays a central role in both development and in adult tissue homeostasis. Hes1 is one of the first known target genes whose transcription is stimulated by Notch signaling[23]. Hes1 can control the activity of cyclin-dependent kinase and inhibit protein transcription, which directly promotes the proliferation of progenitor cells. In embryonic brain tissue, neural progenitor cells also require the proper expression of Hes1. Precursor cells easily differentiate into nerve cells after Hes1 gene inactivation. Without Hes1, neural stem cells prematurely differentiate into neurons. Studies also show that Hes1 plays an important role in endocrine development and blood diseases, including diseases of the thyroid gland and pancreas, and in T cell leukemia[24]. Fibroblast growth factor-signaling controls different biological activities, and is involved in Hes1-mediated self-renewal, which includes proliferation of neural stem/progenitor cells. These studies, as well as studies using Hes1 overexpressing cells show that these genes have a regulatory function in the proliferation and differentiation of nerve precursor cells[25]. Thus, we suggest that the low expression of Hes1 promotes MDSCs to differentiate into neuron-like cells.

In this study, Hes1 protein expression was significantly decreased during the differentiation of MDSCs into neuron-like cells. This result confirmed that the Hes1 protein plays an important role in induction and differentiation *in vitro*. The present study discussed the effect of this pathway on the differentiation of MDSCs into neuron-like cells, and provided a theoretical basis for stem cells in the repair of spinal cord injury. However, further research is required to identify the specific mechanisms involved.

MATERIALS AND METHODS

Design

An *in vitro* based observational experiment.

Time and setting

The experiment was performed at the Liaoning Medical University in China from September 2011 to January 2012.

Materials

Ten healthy, clean, male, Sprague-Dawley rats aged 3 days and weighing 10 ± 2 g were provided by the Laboratory Animal Center of Liaoning Medical University in China (Permit No. SCXK (Liao) 2003-2007). The protocols were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[26].

Methods

Primary culture and identification of MDSCs

Under aseptic conditions, the skeletal muscle of Sprague-Dawley rats was digested with a mixed enzyme solution (0.2 g type II collagenase, 0.48 g type II dispase, and 0.028 g $CaCl_2$ in 100 mL mixed enzyme) for an hour, then filtered using a sieve. After centrifugation (800 r/min, 10 minutes), cells (P1) were resuspended in high glucose Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) with 15% (v/v) fetal bovine serum and maintained in 5% CO_2 at 37°C. With time intervals of 2 (P2), 16 (P3), 24 (P4) and 24 hours (P5), cells were purified by differential adhesion. After cells had been plated for 24 hours, cells were digested using 0.25% (w/v) trypsin for subculture. Passaged cells were identified by anti-desmin (rabbit anti-rat NSE monoclonal antibody) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) immunocytochemical staining.

Cell culture and expression of surface antibodies on the control and experimental groups

After centrifugation at 800 r/min for 10 minutes and

resuspension, P5 cells were divided into two groups and seeded at 4–8 × 10^6/mL. Cells in the control group were incubated in complete medium. Cells in the experimental group were incubated in Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) fetal bovine serum, 20 μg/L nerve growth factor (Sigma, St. Louis, MO, USA), 20 μg/L basic fibroblast growth factor (Sigma) and 1% (v/v) penicillin[23]. After 6 days of culture in the two groups, cells were identified by NSE immunocytochemistry and Hes1 protein immunofluorescence expression.

Immunocytochemistry detection

Sterile coverslips were put into the culture dish along with the cell suspension. After the cells were incubated in a incubator containing 5% CO_2 for 2–3 days and fixed with 4% (w/v) paraformaldehyde for 30 minutes, cells were incubated with 0.3% (v/v) Triton-X 100 for 5 minutes and 3% (v/v) H_2O_2 for 20–30 minutes. Cells were mixed with bovine serum albumin for 20 minutes, and incubated with the primary antibody (rabbit anti-rat NSE monoclonal antibody) (1:100; Santa Cruz Biotechnology) overnight at 4°C. Cells were then incubated with secondary antibody (goat anti-rabbit IgG) (1:200; Zhongshan Goldenbridge Bio-tech Company, Beijing, China) for 2 hours. Cells were incubated with streptavidin-biotin-peroxidase complex (Boster Biological Engineering, Wuhan, China) for 30 minutes and stained with diaminobenzidine (Boster Biological Engineering) for 30 minutes. After re-dyeing, the cells were dehydrated an mounted with hematoxylin, and observed using an inverted phase contrast microscope (Olympus, Tokyo, Japan).

Immunofluorescence cytochemistry

Sterile coverslips were placed into the culture dish along with the cell suspension. Cells were then incubated in an incubator containing 5% CO_2 for 2–3 days, fixed with 4% (w/v) paraformaldehyde for 30 minutes, and soaked in 0.3% (v/v) Triton-X 100 for 5 minutes and 3% (v/v) H_2O_2 for 20–30 minutes. Cells were then incubated in confining liquid for 20 minutes, followed by incubation with rabbit anti-rat Hes1 polyclonal primary antibody (1:100; Santa Cruz Biotechnology) at 4°C overnight. Cells were incubated with goat anti-rabbit IgG/tetramethylrhodamine isothiocyanate (1:200; Zhongshan Goldenbridge Biotech Company) for 2 hours, followed by incubation with streptavidin-biotin-peroxidase complex for 30 minutes. Cells were then stained with diaminobenzidine for 30 minutes, re-dyed with hematoxylin for half a minute and dehydrated with alcohol for 4 minutes progressively (75%–85%–95%–100%). Cells were permeabilized with xylene I and xylene II, both for 10 minutes, and mounted using glycerin buffer and non-fluorescent nail polish. Cells were observed using fluorescence microscopy (Olympus).

Detection of Hes1 protein expression using western blot

MDSCs and differentiated neural-like cells were washed with PBS, detached from the wall of the dish, and centrifuged at 12 000 r/min for 10 minutes at 4°C. The supernatants were stored at –80°C. The protein concentrations were measured using the Lowry method[27], and bovine serum albumin served as a standard. Protein (20 μg) was separated on a 12% (w/v) sodium dodecyl polyacrylamide gel. After electrophoresis, the protein was electrotransferred to nitrocellulose membrane. The membrane was blocked using 3% (w/v) bovine serum albumin for 60 minutes, then washed with tris-buffered saline (TBS; 10 mM Tris, 150 mM NaCl, 3 × 10 minutes). The membrane was soaked in the primary antibody (rabbit anti-rat Hes1 polyclonal antibody) (1:500; Santa Cruz Biotechnology) at 4°C overnight. After being washed with TBS, the membrane was incubated in the secondary antibody (goat anti-rabbit IgG, 1:200; Zhongshan Goldenbridge Bio-tech Company) conjugated to alkaline phosphatase at room temperature for 1–2 hours and washed with TBS (3 × 10 minutes). The membranes were soaked in Nitro-Blue-Tetrazolium/ 5-bromo-4-chloro-3-inodlyl-phosphate colored fluid until the color developed. β-actin was used as the internal control. Protein bands were analyzed using gel automatic analysis imaging software (ImageMaster VDS-CL, Amersham Pharmacia, Uppsala, Sweden) after western blot developing images were scanned. Relative absorbance levels were expressed, and experiments were repeated five times.

Statistical analysis

Data were analyzed using SPSS 16.0 software (SPSS, Chicago, IL, USA), and expressed as the mean ± SD. Experimental and control groups were compared with the two samples *t*-test. A value of $P < 0.05$ was considered statistically significant.

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Author contributions: Xifan Mei was responsible for funding and authorized the present study. Kun Liu, Mina Huang, Zhanpeng Guo, Shiqiang Fang, Jinhao Zeng, Yansong Wang

and Yajiang Yuan designed and performed the present study, and also participated in data analysis and statistical analysis.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animal Ethics Committee of Liaoning Medical University, China.

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