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Original Article

Spinal cord extracts from injured spinal cord impede differentiation of rat embryonic neural stem cells into neurons through regulating Notch signaling pathway

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Abstract: SCI (spinal cord injury) is a complex and serious neurological disease with no efficient treatment. NSC (neural stem cells) have the potential for self-renewal, proliferation and differentiation into all types of nerve cells. The aim of our study is to evaluate the effect of SCE (spinal cord extracts) from injured spinal cord on the differentiation of rat embryonic NSC and to clarify its potential mechanism. Here, NSC were isolated and cultured with SCE. The experiments were divided into four groups, including NSC + sham, NSC + SCE, NSC + SCE + DMSO (dimethyl sulfoxide), NSC + SCE + DAPT (N-[N-(3,5-difluorophenacetyl)-1-alanyl]-S-Phenyl-glycinet-butylester). The Notch1 (notch receptor 1) and Hes1 (hes family bHLH transcription factor 1) mRNA expression was analyzed by qPCR (quantitative real-time PCR) analysis. The protein expression levels of GFAP (glial fibrillary acidic protein) and NSE (nestin) were evaluated by immunofluorescence staining. Cell differentiation of NSC was induced by using neurobasal medium. The results showed that the NSC were successfully identified, and could proliferate to form spherical aggregates and was passaged continuously and steadily *in vitro*. The NSC at fifth generation were positively stained with NSE, and was capable of differentiating into NSE-positive cells and GFAP-positive cells. SCE treatment could upregulate the mRNA expression levels of Notch1 and Hes1, but inhibited the differentiation of NSC into neurons. DAPT could down-regulate the mRNA expression of Notch1 and Hes1 in NSC. Mechanically, DAPT targeting Notch signal pathway could facilitate NSC differentiation into neurons. Together, our data highlighted that SCE suppresses the differentiation of rat embryonic NSC by regulating the Notch signaling pathway, and DAPT treatment can reverse the effect of SCE related differentiation.

Keywords: Neural stem cells, spinal cord extracts, Notch, DAPT, differentiation

Introduction

SCI (spinal cord injury) is a complex and serious neurological disease worldwide [1, 2]. SCI is often caused by traumatic events, including accidental falls, traffic accidents, violence, and sports, which leads to severe sensory, motor, and autonomic dysfunction. To date, there is no efficient neurorestorative therapy for SCI treatment. Emerging data have shown that the activation of progenitor/stem cells is a novel promising strategy for recovery from SCI [3, 4]. For instance, Chen et al. [5] found that E-cadherin regulates biologic behaviors of neural stem cells and promotes motor function recovery following SCI. Tsai et al. [6] demonstrated that bone marrow mesenchymal stem cell treatment promotes SCI repair and functional recovery by activation of autophagy and enhancement of survival-related proteins. Nori et al. [7] reported that human oligodendrogenic neural progenitor cells delivered with chondroitinase ABC facilitate functional repair of chronic SCI.

NSC (neural stem cells) are undifferentiated self-renewing progenitor cells that are able to differentiate into neurons, astrocytes, and oligodendrocytes [8]. Previous studies have confirmed that NSC transplantation is a promising regenerative therapy for various of nervous sys-

tem disorders including SCI [9]. NSC proliferation and differentiation are the two important processes in functional recovery after SCI [10]. Many molecules have been confirmed to be related to NSC proliferation and differentiation [11]; but so far, there remains much to be further clarified. Recently, Yu et al. [12] demonstrated that melatonin promotes proliferation of NSC from adult mouse spinal cord via the PI3K/AKT signaling pathway. Fu et al. [13] suggested that EID1 (EP300-interacting inhibitor of differentiation 1) plays a crucial role in proliferation of NSC. Zhao et al. [14] showed that loss of chromatin modulator Dpy30 compromises proliferation and differentiation of NSC. In addition, Gao et al. [15] found that microRNA-342-5p could function as a downstream effector of Notch signaling to regulate the differentiation of NSC into intermediate neural progenitors and astrocyte commitment. Therefore, a better understanding of the mechanisms whereby NSC-associated functional improvements occur will be important to make NSC transplantation a viable clinical option and lead to the development of targeted therapy.

To evaluate the effect of spinal cord extracts (SCE) from injured spinal cord on the differentiation of rat embryonic NSC and to clarify its potential mechanism, this study initially investigated the expression of the Notch1 (notch receptor 1) and Hes1 (hes family bHLH transcription factor 1) mRNA expression by qPCR (quantitative real-time PCR) analysis. Actually, SCE could upregulate the expression levels of Notch1 and Hes1 mRNA in NSC. Functional analysis showed that SCE inhibited NSC to differentiate into neurons. Mechanically, DAPT (N-{N-(3,5-difluorophenacetyl)-1-alanyl}-S-Phenyl-glycinet-butylester) targeting Notch signal signaling could reverse the effect of SCE related differentiation. Our findings uncover an important role of SCE in impeding the differentiation of NSC into neurons through regulating the Notch signaling pathway.

Materials and methods

Ethics statement

The protocol of animal experiments was conducted with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The study was approved by the Committee on the Ethics of Animal Experiments in The Affiliated Hospital of Southwest Medical University (Luzhou, China).

Primary rat embryonic NSC isolation and culture

The six-week-old female SD (Sprague-Dawley) rats (body weight at 220-250 g) at 14.5 days of gestation were obtained from Cyagen Biosciences Inc. (Guangzhou, China). The mice were housed in SPF (specific pathogen-free) conditions with enough food and water. The primary rat embryonic NSC was isolated according to previous literature [16]. The cells were cultured in DMEM/F12 (Dulbecco's modified Eagle's medium/F12) medium (Thermo Fisher Scientific, Waltham, MA, USA) supplement with 1% B27 (Sigma-Aldrich, MO, USA), 1% N2 (Sigma-Aldrich), 1% Pen-Strep (Thermo Fisher Scientific), 20 ng/ml EGF (epidermal growth factor; PeproTech, Rocky Hill, NJ, USA), and 20 ng/ml bFGF (basic fibroblast growth factor; PeproTech). The cells were maintained at 37°C in a 5% CO_2 humidified atmosphere. The media was replaced every two days during the cell growth based on the density, nutrient consumption rate, and by-product formation rate. The cells were allowed to proliferate to form neurospheres, and observed under an Olympus inverted IX71 microscope (Olympus, Tokyo, Japan).

SCE preparation

60 six-week-old male SD rats (body weight at 220-250 g) were randomly assigned to the NSC + sham, NSC + SCE, NSC + SCE + DMSO (Dimethyl sulfoxide), NSC + SCE + DAPT groups at 15 rats per group. The rats were anesthetized with 10% chloral hydrate (350 mg/kg intraperitoneally) and fixed in situ. The skin and muscle were incised to expose T8-T10 spinal cord. Spinal nerve roots outside of the spinal cord were separated, T8-T10 spinal cord were taken out and immediately placed in sterile PBS (Sigma-Aldrich) at 4°C. Then, the specimen was transferred into a 2 ml grinder and added with 1 m sterile PBS at 4°C. After that, specimen were homogenized and centrifuged at 3 000 rpm for 10 min. Finally, the supernatant was filtered using 0.22 μm syringe filters (Sigma-Aldrich) twice and froze at -20°C.

DAPT treatment

The NSC were cultured and grouped as per above description. The DAPT was obtained

from Sigma-Aldrich Co. LLC (Sigma-Aldrich). For DAPT treatment, 50 μmol/l DAPT was added to the SCE + DAPT group, according to the manufacturer's protocols. An equal dose of DMSO was used as a negative control to add to the SCE + DMSO group. After 48 h of cell culture, the efficiency of Notch signaling pathway knockdown in SCE + DAPT was quantified by qPCR analysis.

Cell differentiation

NSC were cultured with DMEM/F12 medium for 24 h in culture dishes that had been coated with extracellular matrix proteins (Corning, NY, USA) at 37°C overnight. The neuronal differentiation was then induced by changing the medium to neurobasal medium supplemented with 1% B27, 1% N2, 1% Pen-Strep, and 5% FBS (Thermo Fisher Scientific).

qPCR analysis

Total RNA of NSC was extracted using TRIzol reagent (Thermo Fisher Scientific). The cDNAs was synthesized by Oligo-dT primers (Takara, Dalian, Japan) with 1 μg total RNA, according to the manufacturer's protocols. The qPCR assays were performed on ABI PRISM 7000 Sequence Detection System (Applied Biosystems, CA, USA). For determination of the Notch1 and Hes1 mRNA levels, the qPCR was conducted by using the Takara EmeraldAmp PCR Master Mix (Takara). The specific primers for Notch1, Hes1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: Notch1-F, 5'-CGGCTGGTCAGGGAAATCGTGC-3' and Notch1-R, 5'-TGGGTGGGCAGTGGCAGATGTAGG-3'; Hes1-F, 5'-GCGCCGGGCAAGAATAAATG-3' and Hes1-R, 5'-TCGGTGTTAACGCCCTCACAC-3'; GAPDH-F, 5'-GACCCCTTCATTGACCTCAAC-3' and GAPDH-R, 5'-CGCTCCTGGAAGATGGTGAT-3'. Relative levels of Notch1 and Hes1 were normalized to GAPDH, and quantified by the comparative Cq method using the formula $2^{-\Delta\Delta Cq}$ [17].

Immunofluorescence staining

NSC was seeded on poly-L-ornithine-coated slides (Corning) and fixed with 4% paraformaldehyde (Sigma-Aldrich) for 30 min at 37°C, and then washed three times with PBS. Cells were treated with 10% bovine serum (Thermo Fisher Scientific) and 0.5% Triton X-100 (Sigma-Aldrich) for 60 min at 37°C. Subsequently, cells were washed three times with PBS, and incubated with mouse anti-NSE (nestin) primary antibody (1:200, #ab218388; Abcam, CA, USA) and mouse anti-GFAP (glial fibrillary acidic protein) primary antibody (1:400, #ab10062; Abcam, CA, USA) overnight at 4°C. After washing with PBS, cells were incubated with the Cy3 (1:500, #ab97035) or FITC (1:500, #ab6785)-labeled goat anti-mouse IgG (Abcam) for 2 h at 37°C. After washing with PBS, cells were dyed with DAPI (Abcam) for 2 min at 37°C. The images were obtained using an Olympus model BX41 fluorescence microscope (Olympus, Tokyo, Japan).

Statistical analysis

All of the experiments were conducted at least three times. Statistical analyses were conducted using SPSS 17.0 software (SPSS Statistics Inc., IL, USA). The data were presented as the mean ± standard deviation (SD). The differences between two groups were analyzed using two-sided Student t-test. $P < 0.05$ was considered significant.

Results

Identification of primary rat embryonic NSC

A large number of primary rat embryonic NSC were observed at 24 h of cell cultivation, and the cells may appear to be split and renewed (**Figure 1A**). Several dozens of translucent, high-refractive, spherical or string-shaped cell aggregates could be seen at 2 days of cell culture (**Figure 1B**). NSC is obviously larger, spherical, and had a strong proliferative capacity at 4 days of cell culture (**Figure 1C**). In cell culture for 8 days, the volume of NSC was significantly larger than that of the cell culture for 4 days. The refractive index of NSC was poor, and its central part was shown as tan (**Figure 1D**). A large and well-sized cloned neurosphere can be observed in the NSC after the fifth generation (**Figure 1E**). After 5 days of NSC differentiation, the neurospheres were flattened, and a large number of cells with different shapes were formed around the neurosphere, and were connected to each other to form an intricate network structure (**Figure 1F**). Immunofluorescence staining showed that the NSC at fifth generation were positively stained with NSE (**Figure 2A**), and were capable of differentiating into NSE-positive cells and GFAP-positive cells

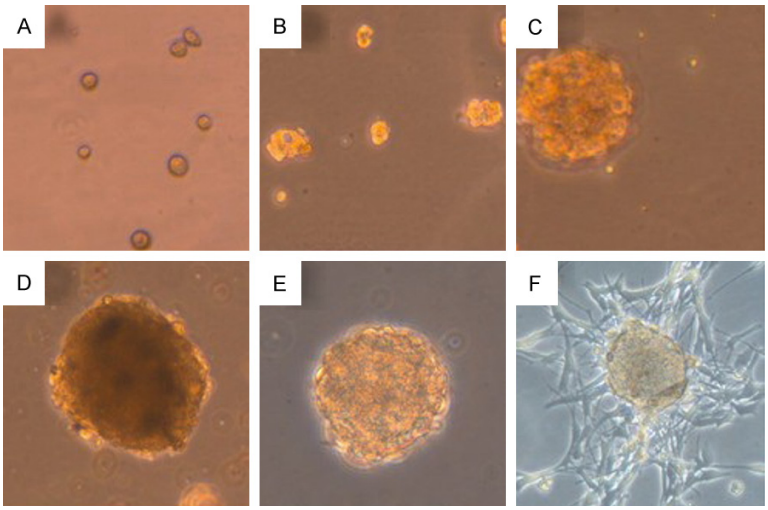

Figure 1. Identification of primary rat embryonic NSC. A-D. Images of primary rat embryonic NSC after 24 h, 2 days, 4 days, 8 days of cell cultivation (×100 magnification). E. A large and well-sized clonal neurosphere can be observed in the NSC after the fifth generation (×200 magnification). F. After 5 days of NSC differentiation, a large number of cells with different shapes formed around the neurosphere, and were connected to each other to form an intricate network structure (×200 magnification). NSC: neural stem cells.

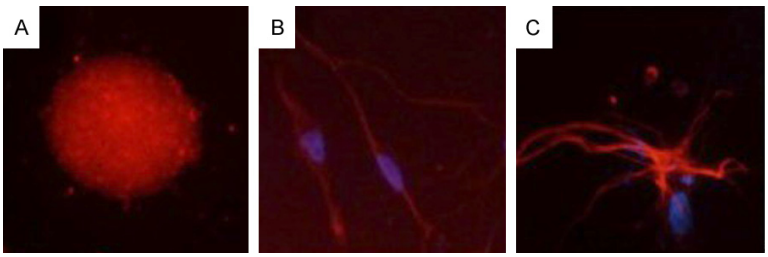

Figure 2. Immunofluorescence staining for analysis of the expression and location of GFAP and NSE in NSC. A. NSC at the fifth generation were positively stained with NSE (red, ×100 magnification). B, C. After 5 days of neuronal differentiation, NSE were capable of differentiating into NSE-positive cells and GFAP-positive cells (red, ×200 magnification). Nucleus was stained with DAPI (blue). NSE: nestin; GFAP: glial fibrillary acidic protein.

(**Figure 2B** and **2C**). The data demonstrated that the NSC were successfully identified, and could proliferate to form spherical aggregates and be passaged continuously and steadily *in vitro*.

SCE impedes the differentiation of NSC into neurons and increases mRNA expression levels of Notch1 and Hes1

The percentage of NSE-positive cells was lower in the NSC + SCE group than that in the NSC + sham group, while the percentage of GFAP-positive cells had no difference between the NSC + SCE group and NSC + sham group (**Figure 3A**, $P < 0.05$). Further, Notch1 and Hes1 mRNA expression were analyzed by qPCR analysis. The results showed that the expression levels of Notch1 and Hes1 mRNA in the NSC + SCE group were significantly upregulated compared with the NSC + sham group (**Figure 3B**, $P < 0.05$). The data demonstrated that SCE suppresses the differentiation of NSC into neurons and increases the Notch1 and Hes1 mRNA expression.

DAPT targeting Notch signaling pathway induces NSC differentiation into neurons

To further identify the potential mechanism of SCE on NSC related differentiation, DAPT, an effective inhibitor of the Notch signaling pathway, was applied to knock down the Notch signaling pathway. qPCR analysis showed that the expression levels of Notch1 and Hes1 mRNA in the NSC + SCE + DAPT group were downregulated compared with the NSC + SCE + DMSO group (**Figure 4A**, $P < 0.05$). Mechanically, the percentage of NSE-positive cells was higher in the NSC + SCE + DAPT group than that in the NSC + SCE + DMSO group, while the percentage of GFAP-positive cells showed no difference between the NSC + SCE + DAPT group and the NSC + SCE + DMSO group (**Figure 4B**, $P < 0.05$). The data demonstrated that DAPT treatment reverses the inhibition of SCE on NSC-related differentiation, indicating SCE impedes differentiation of rat embryonic NSC into neurons by regulating the Notch signaling pathway.

Discussion

Spinal cord injury (SCI) has been thought to be an irreversible type of neurological disease [18]. Evidence has shown a desire to minimize secondary injury and promote neurological recovery from SCI [19]. Recently, neural stem cell (NSC) differentiation after SCI was shown

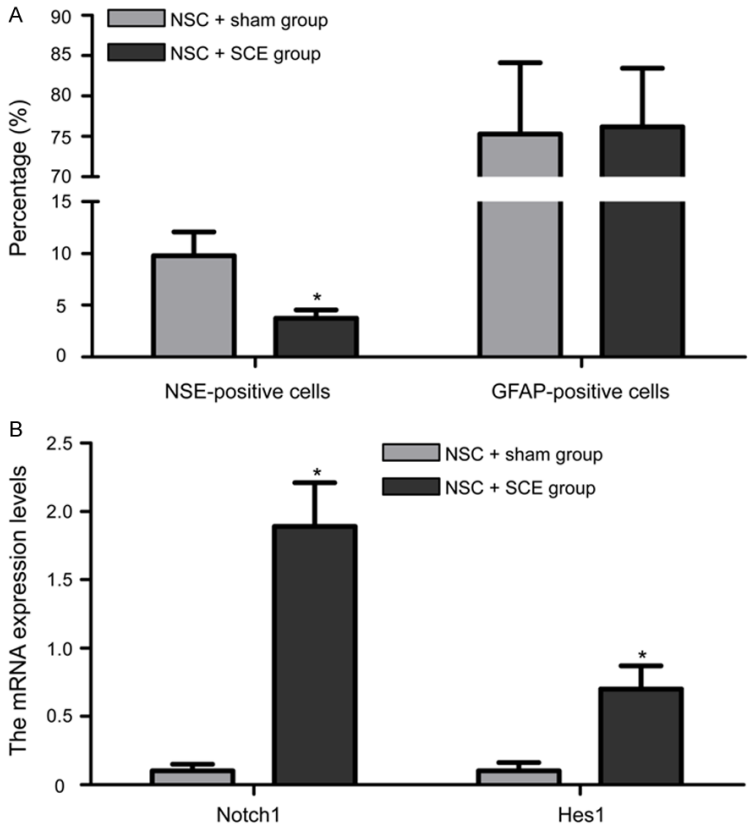

Figure 3. Spinal cord extract (SCE) impedes the differentiation of NSC into neurons and increases the mRNA expression levels of Notch1 and Hes1. A. The percentages of NSE and GFAP-positive cells in the NSC + SCE group and the NSC + sham group. B. qPCR analysis showed that the expression levels of Notch1 and Hes1 mRNA in the NSC + SCE group were significantly upregulated compared with the NSC + sham group. SCE: spinal cord extracts; Notch1: notch receptor 1; Hes1: hes family bHLH transcription factor 1; qPCR: real-time PCR. *$P < 0.05$.

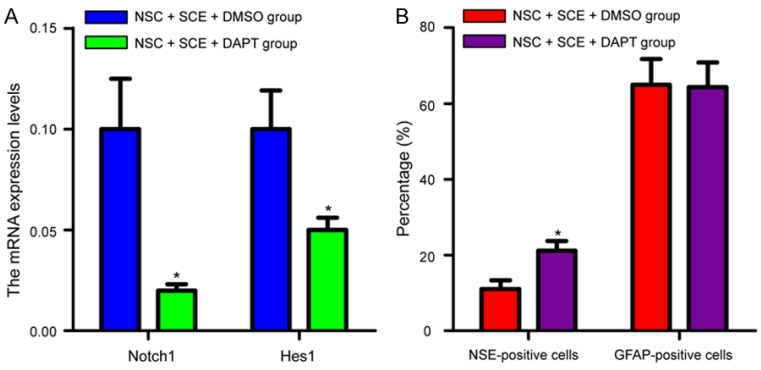

Figure 4. DAPT targeting Notch signal pathway induces NSC differentiation into neurons. A. qPCR analysis of Notch1 and Hes1 mRNA expression in the NSC + SCE + DAPT group and the NSC + SCE + DMSO group. B. The percentages of NSE and GFAP-positive cells in the NSC + SCE + DAPT group and the NSC + SCE + DMSO group. DAPT: N-{N-(3,5-difluorophenacetyl)-1-alanyl}-S-Phenyl-glycinet-butylester; DMSO: dimethyl sulfoxide. *$P < 0.05$.

to play an important role in functional recovery from neurological dysfunction [10]. The differentiation of NSC is regulated by multiple factors, such as many coding genes [20], miRNAs [15], traditional Chinese medicine [21], and hormones [12]. However, the exact mechanism that efficiently activates endogenous NSC for functional recovery after SCI remains elusive. In our study, the role and underlying mechanism of spinal cord extracts (SCE) on differentiation were investigated. Our data suggested that SCE impeded differentiation of rat embryonic NSC into neurons by regulating the Notch signaling pathway.

Here, the embryonic NSC were successfully isolated from six-week-old female SD rats at 14.5 days of gestation. SCE can simulate the microenvironment after SCI when it is cultured with NSC. After 5 days of NSC differentiation, the neurosphere was flattened and a large number of cells with different shapes were formed around the neurosphere. These cells were connected to each other to form an intricate network structure. Immunofluorescence staining showed that the NSC at fifth generation were positively detected with NSE, and were capable of differentiating into NSE-positive cells and GFAP-positive cells. Furthermore, the current study has shown that SCE treatment decreased the percentage of NSE-positive cells and elevated the mRNA expression levels of Notch1 and Hes1 in NSC. The data demonstrated that SCE could impede rat embryonic NSC differentiation into neurons *in vitro*, which was consistent with the results of *in vivo* studies from Horky et al. [22] and Mothe et al. [23]. It can be concluded that

SCE from the injured spinal cord could reflect the microenvironment after SCI to some extent which might regulate NSC differentiation into neurons.

Notch is a fate signal integrator that is universally utilized in stem cells during development, allowing cells to maintain self-renewal [24]. The Notch signaling pathway has been reported to play multiple roles during the development of the central nervous system [25]. The Notch signaling pathway is thought to maintain "stemness" and to prevent exit from the cell cycle and maturation [26]. Recent studies have provided abundant evidence that targets of Notch signaling work together to prevent terminal differentiation and to preserve a pool of stem cells [27]. For example, Notch inhibitors from Calotropis gigantea induce neuronal differentiation of NSC [28]. Notch signaling is essential for maturation, self-renewal, and tri-differentiation of *in vitro* derived human NSC [29]. MicroRNA-9 stimulation enhances the differentiation of NSC with zoanthamine by regulating Notch signaling [30]. DAPT is an effective inhibitor of the Notch signaling pathway, which delivers cell signals through receptors and ligands of adjacent cells and influences the morphology and neural differentiation of NSC [31]. To further identify the potential mechanism of SCE on NSC related differentiation, the DAPT was applied to target Notch signal pathway. Here, our results showed for the first time that DAPT treatment could reverse the inhibition of SCE on NSC differentiation into neurons.

In summary, our report provides novel evidence that SCE impedes differentiation of rat embryonic NSC into neurons by regulating the Notch signaling pathway, indicating that SCE combined with NSC may be a novel promising therapy for SCI treatment.

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Disclosure of conflict of interest

None.

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