Overexpression of Suppressors of Cytokine Signaling 1 Promotes the Neuronal Differentiation of C17.2 Neural Stem Cells

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Abstract
Objective: To investigate the role and mechanism of suppressor of cytokine signaling 1 (SOCS1) in the regulation and differentiation of C17.2 neural stem cells (NSCs). Methods: In this study, lentiviral (LV)-SOCS1-enhanced green fluorescent protein (EGFP) was constructed and transfected into C17.2 NSCs. There were three groups of C17.2 NSCs: LV-SOCS1-EGFP, LV-EGFP, and phosphate-buffered saline (PBS). The expression levels of microtubule-associated protein 2 (MAP2), glial fibrillary acidic protein (GFAP), myelin basic protein (MBP), nestin, and β-tubulin III in C17.2 NSCs were analyzed by reverse transcription–polymerase chain reaction (RT-PCR), immunocytochemistry, and western blot. In addition, the phosphorylation level of Jaks/Stats family members in C17.2 NSCs were analyzed by western blot. Moreover, the morphological changes of C17.2 NSCs after transfection were observed by light microscopy. Results: The gene expression of MAP2 increased significantly and the gene expression of nestin decreased significantly in C17.2 NSCs transfected with LV-SOCS1-EGFP. Some C17.2 NSCs underwent prominent neuronal morphological changes and expressed β-tubulin III after LV-SOCS1-EGFP transfection. The number of positive cells for β-tubulin III immunocytochemical staining and β-tubulin III protein expression in C17.2 NSCs after LV-SOCS1-EGFP transfection were both more than those after LV-EGFP transfection or PBS treatment. The phosphorylation levels of Jak2 and Stat3 but not Jak3 in C17.2 NSCs were inhibited by SOCS1 overexpression. Conclusion: Overexpression of SOCS1 in C17.2 NSCs promotes the generation of neurons, which is likely mediated by the negative feedback inhibition of Jak2 and Stat3. This study is the first to provide evidence that SOCS1 is involved in the regulation of neurogenesis.
Introduction

C17.2 neural stem cells (C17.2 NSCs) are V-myc gene-transferred neural stem cells, which are obtained from the mouse cerebellum. They can self-copy and proliferate massively in vitro, and maintain their cellular properties for more than 10 weeks of subculture with the ability to differentiate into neurons and glial cells in response to different factors [1]. C17.2 NSCs are also good candidate cells for genetic engineering. If they are transfected with exogenous genes, they can stably express the products of exogenous genes. Simultaneously, the growth of C17.2 NSCs is not obviously affected. These biological characteristics of C17.2 NSCs make them a reliable system for use in experiments with regard to the differentiation properties of NSCs and make them a good transplantation tool for the future treatment of central nervous system (CNS) injury [2].

Neuronal differentiation of NSCs is a complex process that involves the interaction of many intrinsic and environmental cues. Many previous studies have focused on the regulation of extrinsic factors such as cytokines, growth factors, cell interactions, extracellular matrix, etc. to control the differentiation of NSCs [3]. However, research relating to the endogenous regulation of NSCs such as intracellular structural proteins, transcription factors, transcripts, telomeres, etc. is less reported [4]. Therefore, studying the roles and mechanisms underlying the endogenous regulation of NSCs may be beneficial and will hopefully provide us with new ideas for the treatment of CNS injury in the future.

Suppressors of cytokine signaling (SOCS) proteins have been shown to have a wide range of functions and complex regulatory mechanisms during intracellular signal transduction [5]. The SOCS family consists of eight members, cytokine inducible SH2-containing protein (CIS) and the SOCS 1–7 proteins [6]. Some members of the SOCS family have been shown to be involved in the process of neuronal differentiation [7]. Overexpression of SOCS2 seems to directly regulate the ability of a neural progenitor cell to differentiate into a neuron and promotes neuronal differentiation by blocking the growth hormone-mediated downregulation of Ngn1 [8]. Overexpression of SOCS3 induces neurogenesis and inhibits astroglialogenesis in NSCs; and SOCS3 may indirectly regulate the differentiation of NSCs into neurons through the paracrine system without affecting JAK/STAT signaling [9-10]. Overexpression of SOCS6 in NSCs promotes neurite outgrowth via the JAK2/STAT5-mediated signaling pathway, which involves negative feedback inhibition [11].

The SOCS1 protein is an extensively studied SOCS family member [12]; however, its function during differentiation of NSCs, especially with regard to neuronal differentiation, remains unclear. It has been shown that SOCS1 is expressed in the nervous system throughout development [13]. Reducing SOCS1 upregulation may be an additional way in which cyclic nucleotides augment cytokine-induced regenerative responses in the injured CNS [14]. SOCS1 can also regulate interferon gamma-mediated survival of sensory neurons [15]. Based on these studies, we hypothesized that SOCS1 is involved in the regulation of neuron generation. To testify the hypothesis, we employed the lentiviral transfection method to overexpress SOCS1 in C17.2 NSCs and used reverse transcription–polymerase chain reaction (RT-PCR), western blotting, and immunofluorescence staining methods to observe gene expression of neuronal and glial cell markers as well as protein expression of neuronal cell markers and some members of the JAK/STAT family in order to partly elucidate the effects and mechanisms underlying SOCS1 action on the neuronal differentiation of NSCs.

Materials and Methods

Lentiviral (LV)-SOCS1-enhanced green fluorescent protein (EGFP) construction and viral production

Human SOCS1 cDNA (NM_024509, Genecopoeia, USA) was amplified by PCR (SOCS1-Forward: CGGGAATTCTAGTGAGACAAACCAAGGTTG; SOCS1-Reverse: ACGGGATTCCTCAAATGGAAGGGGAAGGAG), and amplified fragments were cloned into a cleaved LV transfer vector (System Biosciences, USA). The resulting LV plasmids were sequenced and identified by PCR. The recombinant LV encoded SOCS1 as
well as EGFP. All recombinant lentiviruses were produced through transient transfection of the human embryonic kidney cell line (293T) according to standard protocols [16]. Viruses were concentrated by ultracentrifugation, aliquoted, and stored at -80 °C. Viral titers were calculated by infecting 293T cells grown in a 96-well plate with serially diluted virus and counting the number of fluorescent cells in the final two wells. This number was divided by the dilution to obtain the virus titer value. Titers were expressed as transducing units (TU)/mL.

**Neural Stem Cell Culture—Mouse immortalized NSC line**

C17.2 NSCs (Shanghai University, P. R. China) were originally described by Snyder et al [1]. The C17.2 NSCs were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen, USA) supplemented with 10% fetal calf serum (Hyclone, USA), 5% horse serum (Invitrogen), and 2 mM glutamine (Invitrogen) in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C and split when the cells reached 90% confluency.

**Transduction of C17.2 NSCs with LV vectors**

C17.2 NSCs were seeded in 6-well plates 1 day before transfection, and transfection was performed when the cell density reached 30–50% per well. LV-SOCS1-EGFP or LV-EGFP was then added at a multiplicity of infection (MOI) of 50, and Polybrene (Sigma, USA) was added at a concentration of 5 μg/mL to promote infection. Cells were incubated for 24 h. The medium was then replaced. LV-SOCS1-EGFP or LV-EGFP was added again, and the cells were incubated at 37 °C for 48 h. Some C17.2 NSCs were treated only with phosphate-buffered saline (PBS) as the negative control. After the total number of cells and the cells with green fluorescence were counted in five random fields, the transduction efficiency of NSCs was determined by calculating the percentage of cells with green fluorescence out of the total number of cells, with untransfected NSCs serving as a control.

**Western blot analysis**

Western blotting was performed according to standard procedures. Briefly, after rinsing with cold D-Hanks buffer, C17.2 NSCs were collected and lysed. Protein samples were extracted and measured using a BCA Protein Assay kit (Abcam, Britain). Approximately 32 µL of protein in 8 µL of 5× loading buffer (Santa Cruz, USA) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (350 mA, 120 min; Santa Cruz, USA). Membranes were blocked with 5% fat-free milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) and were subsequently incubated with the following primary antibodies including rabbit anti-SOCS1 polyclonal antibody, (CST, USA), mouse anti-β-tubulin III monoclonal antibody (Chemicon, USA), rabbit anti-JAK2 polyclonal antibody, rabbit anti-JAK3 polyclonal antibody, rabbit anti-STAT3 polyclonal antibody, rabbit anti-p-JAK2 polyclonal antibody, rabbit anti-p-JAK3 polyclonal antibody, and rabbit anti-p-STAT3 polyclonal antibody (Santa Cruz, USA). The working concentrations of these primary antibodies were all 1:200, and these antibodies were incubated at 4 °C overnight. Then, the membranes were washed with TBST and incubated for 2 h with horseradish peroxidase-conjugated secondary antibodies (1:1000, KPL, USA). The membranes were finally developed using an electrochemiluminescence kit (Perkin Elmer, USA). Band intensity was quantified by gel densitometry with the Gel Image Analysis System. Protein phosphorylation was normalized against total protein band densitometry on an individual basis.

**RT-PCR**

Cells were washed with PBS, and total RNA was extracted with the aid of TRIzol reagent (Invitrogen). The primers were designed according to the manufacturers’ protocols (see Table 1).

The reverse transcription reaction of 2 μg of RNA was carried out using MMLV reverse transcriptase (Invitrogen, Cat No: 28025-013) and the relevant reagents, according to the manufacturer’s instructions. The total reaction mixture was 20 μL. The PCR of 1 μL of RT product was performed using tag DNA polymerase (Fermentas, Cat No: EP0402, Canada). The synthesized cDNA was amplified under the following conditions: initial denaturation at 94 °C for 3 min; denaturation at 94 °C for 30 s; annealing at 56 °C for 30 s; and extension at 72 °C for 60 s for 24 cycles. Amplification products were identified by size and confirmed by DNA sequencing. PCR products were electrophoresed in 2% agarose gel and stained with ethidium bromide. The relative gene expression was semi-quantified by using AlphaEaseFC computer software (Cell Biosciences, USA), normalized with GAPDH as the internal control, and compared with the dimethyl sulfoxide vehicle control.
Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (diluted in 0.1 mM PBS) for 10 min at room temperature, permeabilized with 2% Triton X-100 for 10 min, and then washed three times with 0.01 mM PBS. The cells were then pre-incubated with 10% bovine serum albumin (diluted in 0.1 mM PBS) for 1 h at room temperature to block nonspecific binding with the primary antibodies: goat anti-nestin polyclonal antibody and mouse anti-β-tubulin III monoclonal antibody (Chemicon, USA) were diluted (in 0.1 mM PBS) to 1:200 and 1:50, respectively. After being incubated at 4 °C overnight, the cells were washed again with PBS and incubated in the dark for 1 h with secondary antibodies: fluorescein isothiocyanate for nestin and tetramethylrhodamine isothiocyanate for β-tubulin III; both working concentration were 1:200. Positive staining was examined as green and red fluorescence, respectively, under a microscope.

Statistical analysis

Cells that were positive for β-tubulin III were counted in 10 individual random nonoverlapping fields during each experiment. All values are shown as means ± standard deviation. Differences between two groups were determined with the unpaired Student’s t-test. Analysis of variance was used for multiple comparisons. A P < 0.05 was considered statistically significant.

Results

C17.2 NSCs were nestin-positive

We observed that normally cultured C17.2 NSCs were shuttle-like or irregular shaped without recognizable soma parts, and they had no obvious long tube-like processes (Fig. 1a). We detected the expression of nestin, an NSC marker, in C17.2 NSCs using an anti-nestin antibody. As shown in Figure 1b and 1c, most of the cultured C17.2 NSCs were nestin-positive.

Verification of LV-SOCS1-EGFP and LV-EGFP transfection

NSCs are difficult to transfect; thus, to analyze the transfection efficacy, we used an LV vector for transfection with an EGFP tag, which showed green fluorescence. We found that the transfection rate reached approximately 80% (Fig. 2a and 2b) after optimization (shown in methods). Using western blotting analysis, an obvious increase of 2.06-fold and 2.29-fold in SOCS1 protein was observed in the LV-SOCS1-EGFP group, respectively, when compared to that in the LV-EGFP group and PBS group (Fig. 3a and 3b; P < 0.001). This result suggested that the LV vector was suitable for use in this study.

C17.2 NSCs undergo prominent neuronal morphological changes after transfection

We examined cells from the first day to the third day after transfection. One day after transfection, we found that some cells had undergone a morphological change: The soma parts became recognizable, and an outgrowth of small neurite-like organelles was found on
the cell surfaces (Fig. 5d). Two days after transfection, the soma parts of these cells changed from shuttle-like to round, and the neurite-like structures extended further (Fig. 5e). Three days after transfection, the somas became more rounded and the outgrowths began to branch, forming cells which morphologically resembled typical neurons (Fig. 5f).

An increased gene expression of Map2 and a decreased gene expression of nestin in C17.2 NSCs transfected with LV-SOCS1-EGFP, showing differentiation into neurons at the transcriptional level

Three days after transfection, the mRNA levels of several specific markers of different lineages of nerve cells were analyzed by RT-PCR (Fig. 4a). We found that the mRNA expression of microtubule-associated protein 2 (Map2), a mature neuron marker, in the LV-SOCS1-EGFP group was increased significantly by 2.91-fold and 2.59-fold when compared to that in the LV-EGFP group and PBS group, respectively (Fig. 4b; P < 0.001). On the other hand, the mRNA expression of nestin in the LV-SOCS1-EGFP group decreased significantly by 0.23-fold and 0.25-fold when compared to that in the LV-EGFP group and PBS group, respectively (Fig. 4e). In contrast, the mRNA expression of myelin basic protein (mbp), an oligodendrocyte marker, and glial fibrillary acidic protein (gfap), an astrocyte marker, remained at similar levels in the LV-SOCS1-EGFP group when compared to the levels in the other two groups (Fig. 4c and 4d). These findings at the transcriptional level suggested that overexpression of SOCS1 could promote the differentiation of C17.2 NSCs towards a neuronal lineage.

C17.2 NSCs can differentiate into neurons at the post-transcriptional level

Three days after transfection, anti-β-tubulin III antibody was used to stain the differentiated cells. β-tubulin III, a neural marker, is a protein expressed during the axonal
outgrowth, and it is useful to detect the growth of an axon-like projection [17]. After transfection with LV-SOCS1-EGFP was performed, we found that almost all the cells with morphological changes were β-tubulin III-positive. However, the cells without morphological
changes were not seen to be β-tubulin III-positive (Fig. 6). Furthermore, the percentage of β-tubulin III-positive cells increased from less than 1% in the PBS group (0.65 ± 0.37%) and LV-EGFP group (0.71 ± 0.42%) to 10% in the LV-SOCS1-EGFP group (10.05 ± 0.69%, P < 0.01). Moreover, the western blot results revealed that the expression of β-tubulin III protein was elevated by 3.11-fold and 3.03-fold in the LV-SOCS1-EGFP group when compared to that in the LV-EGFP group and PBS group, respectively (Fig. 7; P < 0.001). These findings indicated that overexpression of SOCS1 promoted the differentiation of C17.2 NSCs towards a neuronal lineage at the post-transcriptional level.

SOCS1 inhibited the phosphorylation level of JAK2 and STAT3 but not JAK3 in C17.2 NSCs

SOCS1 protein is one of the feedback inhibitors of the JAK/STAT signaling pathway [12]. It has been shown that some of the JAK/STAT family members modulate the differentiation of NSCs [18], and it is possible that they mediate the stimulating effect of SOCS1 on the differentiation of NSCs. Thus, in this study, to investigate the effect of the negative feedback inhibition of the overexpression of SOCS1 on the JAK/STAT signaling pathway in the C17.2 NSCs, we observed the phosphorylation protein levels of JAK2, JAK3, and STAT3 in C17.2 NSCs 3 days after transfection using the western blotting method (Fig. 8a). We found that the phosphorylation level of JAK2 in the LV-SOCS1-EGFP group was decreased by 0.41-fold and 0.44-fold when compared to that in the LV-EGFP group and PBS group, respectively (Fig. 8b; P < 0.001). Similarly, the phosphorylation level of STAT3 in the LV-SOCS1-EGFP group was decreased by 0.41-fold and 0.44-fold when compared to that in the LV-EGFP group and PBS group, respectively (Fig. 8d; P < 0.001). In contrast, the phosphorylation level of JAK3 in the LV-SOCS1-EGFP group was increased by 2.27-fold and 2.12-fold when compared to that in
These findings indicated that overexpression of SOCS1 could inhibit the phosphorylation level of JAK2 and STAT3 but not JAK3 in C17.2 NSCs.

**Fig. 6.** β-tubulin III immunofluorescence staining for C17.2 cells. (a-d) C17.2 cells without morphological changes in the PBS group were not stained with anti-β-tubulin III. (e-h) C17.2 cells without morphological changes in the LV-EGFP group were not stained with anti-β-tubulin III. (i-l) C17.2 cells with morphological changes in the LV-SOCS1-EGFP group were stained with anti-β-tubulin III (red fluorescence).

**Fig. 7.** Western blot analysis for β-tubulin III. (a) Representative western blot bands from the three groups. (b) Quantitative results of β-tubulin III in the three groups. *P < 0.001, compared with the LV-EGFP and PBS groups.

the LV-EGFP group and PBS group, respectively (Fig. 8c; P < 0.001). These findings indicated that overexpression of SOCS1 could inhibit the phosphorylation level of JAK2 and STAT3 but not JAK3 in C17.2 NSCs.
Fig. 8. Western blot analysis for JAK2, JAK3, and STAT3. (a) Representative western blot bands from the total proteins of JAK2, JAK3, and STAT3 and the respective phosphorylated proteins in the three groups. (b) Quantitative results of the expression of p-JAK2 in the three groups. (c) Quantitative results of the expression of p-JAK3 in the three groups. (d) Quantitative results of the expression of p-STAT3 in the three groups. *P < 0.001, compared with the LV-EGFP and PBS groups.

Discussion

In the present study, we verified that C17.2 NSCs were nestin-positive and that these cells had relatively high transfection rates with LV-SOCS1-EGFP. We showed that SOCS1 induced a decreased gene expression of the NSC marker nestin and an increased gene expression of the neuronal marker Map2 in C17.2 NSCs with overexpression of SOCS1; however, no changes were observed in the gene expression of mbp and gfap in C17.2 NSCs with and without transfection of LV-SOCS1-EGFP. Moreover, we found that the morphology of the C17.2 NSCs changed gradually, with the cell morphology resembling typical neurons after transfection with LV-SOCS1-EGFP. Almost all of these cells were β-tubulin III-positive after transfection, with no positive signals in other cells. The number of β-tubulin III-positive cells in the LV-SOCS1-EGFP group increased obviously when compared to that in the PBS group and LV-EGFP group. The remaining cells had normal C17.2 NSC morphology and were nestin-positive. Furthermore, the western blotting results also confirmed an increased expression of β-tubulin III protein in the LV-SOCS1-EGFP group when compared to that in the LV-EGFP group and PBS group. Taken together, these results indicated that overexpression of SOCS1 can result in SOCS1 activity that is not relevant at physiological concentrations and that the overexpression of SOCS1 can promote C17.2 NSCs to differentiate into neurons at both the gene and protein levels. Similar to the other family members SOCS2, SOCS3, and SOCS6, SOCS1 has also been shown to modulate neurogenesis. To the best of our knowledge, for the first time, the role of SOCS1 in regulating neurogenesis has been demonstrated in vitro.

It has been shown that SOCS1 protein is one of the feedback inhibitors of the JAK/STAT signaling pathway and that SOCS1 plays its biological effects of regulating cytokines via the JAK/STAT pathway [18]. To further understand the mechanisms underlying SOCS1 promotion of neural differentiation, we examined the expression of JAK/STAT in transfected and nontransfected C17.2 NSCs. The JAK/STAT family members modulate gene expression during the different stages of brain maturation [19]. Among these family
members, JAK2, JAK3, and STAT3 have all been reported to be involved in the modulation of NSC differentiation. Inhibition of JAK2 signaling in NSCs can suppress astrogliogenesis in NSCs by upregulating both notch1 and notch3 mRNA expression [9], which suggests that more NSCs will probably differentiate into neurons and oligodendrocytes under these conditions. Additionally, inhibition of JAK2 signaling can mediate the neuronal differentiation of NSCs induced by SOCS6 [11], whereas knock-down of JAK3 signaling induces neuronal differentiation accompanied by neurite growth in NSCs [20]. Furthermore, suppression of STAT3 directly induces neurogenesis and inhibits astrogliogenesis in NSCs [21-22]. It is known that SOCS1 is one of the negative regulating factors of the JAK/STAT signaling pathway. SOCS1 can bind with the tyrosine residues of target protein phosphorylation via its SH2 domain, which results in the inactivation of the N-terminus of JAK kinase and inhibition of signal transduction; and the N-terminus of SOCS1 has a kinase inhibitory region (KIR) that can directly inhibit JAK tyrosine kinase activity [23]. Moreover, the promoter of SOCS1 contains the binding sites of STAT1, STAT3, and STAT6, which inhibit binding of the STATs at the receptor sites [24]. Therefore, based on the above reports, we hypothesized that SOCS1 participates in the process of neuronal differentiation of C17.2 NSCs probably via inhibiting the JAK2, JAK3, and STAT3 pathway. To confirm our assumption, we used western blotting to observe the protein expression of phosphorylated JAK2, JAK3, and STAT3. In the present study, we observed a decreased phosphorylation level of JAK2 and STAT3 and an unexpected increased phosphorylation level of JAK3 in the LV-SOCS1-EGFP group when compared to those in the LV-EGFP group and PBS group. These findings indicated that overexpression of SOCS1 could selectively inhibit the phosphorylation level of JAK2 and STAT3 in C17.2 NSCs and probably contribute to the neuronal differentiation of C17.2 NSCs.

Previous studies have addressed the fact that the inhibition of JAK3 can induce neurogenesis in NSCs [21]; however, in our study, the phosphorylation level of JAK3 was not suppressed by SOCS1 during neurogenesis. Moreover, to date, no evidence has shown that an increased phosphorylation level of JAK3 induces the neuronal differentiation of NSCs. It is possible that JAK3 is not directly involved in the negative feedback inhibition of SOCS1 after transfection and may be indirectly relevant to the neuronal differentiation of C17.2 NSCs induced by SOCS1. This result implicates the complicated roles and functions of JAK3 in neurogenesis. Furthermore, a cascade association between JAK2 and STAT3 has not been confirmed in the process of neuronal differentiation of NSCs yet. SOCS1 also probably regulates neuronal differentiation via inhibition of JAK2 and STAT3, respectively. In addition, other members of the JAK/STAT pathway may be inhibited by SOCS1 during the differentiation of C17.2 NSCs. Future studies are required to further understand the mechanisms underlying the differentiation of C17.2 NSCs regulated by SOCS1.

In conclusion, overexpression of SOCS1 can promote C17.2 NSCs to differentiate into neurons, and this effect is probably via the inhibition of JAK2 and STAT3 signal transduction. This study not only reveals the function of SOCS1 in the differentiation of NSCs, but it also provides a new intracellular regulatory site and drug target for the research of NSCs and the treatment of CNS injury in the future.

References


