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Human Umbilical Cord Mesenchymal Stem Cells-Secreted TSG-6 Is Anti-Inflammatory and Promote Tissue Repair After Spinal Cord Injury

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Abstract

Spinal cord injury (SCI) causes patients paralysis and hard to recover. The therapeutic effects of current clinical drugs are accompanied by side effects. In recent years, stem cell therapy has attracted the attention of researchers. Human umbilical cord mesenchymal stem cells (hucMSCs) have been widely used in various diseases due to their excellent paracrine function. TNF-stimulated gene 6 (TSG-6), a secretion factor of stem cells, may play an important role in hucMSCs in the treatment of SCI. So we conducted an experiment to explore its effect. We first observed that the expression of TSG-6 increased in SCI rats after injected with hucMSCs. Then, we used siRNA to knowdown the expression of TSG-6. We treated SCI rats with TSG-6-knockdown hucMSCs. Without TSG-6 expression, hucMSCs treatment made the tissue recovery worse and the number of Nissl bodies less. Meanwhile, neutrophils infiltrated more in the damaged parts. Our research also proved that TSG-6 may help demyelination recovering and alleviate astrocytes gathering in the injury sites. Our study revealed that hucMSCs secreted TSG-6 may decrease the degeneration of myelin sheath, reduce inflammation, decrease neuron loss and promote tissue repair. These results provided a new therapeutic factor for the treatment of SCI.

Keywords

spinal cord injury, TSG-6, hucMSCs, inflammation

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Backgrounds

Spinal cord injury (SCI) may cause paralysis below injured segments and cause various complications (Harness et al., 2008; Ahuja et al., 2017; Granier et al., 2020). As for now, more than 72 million people (Eckert and Martin, 2017, 2019) had paraplegia which seriously affects the life quality of patients. SCI brings expensive treatment costs and care costs to patients and society.

There are two forms of SCI: primary SCI and secondary SCI (Zhang et al., 2020). The former is caused by external forces. The latter refers to the edema and microenvironment full of inflammatory factors after acute SCI (Witiw and Fehlings, 2015; Bradbury and Burnside, 2019). After SCI, there would be excessive immune response, oxidative stress, neuronal necrosis and axon degeneration in injury parts (Li et al., 2017).

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Apoptosis neurons release inflammatory factors (TNF-α, IL-6), which cause a large number of immune cells or glial cells gathering in damaged central nervous system (CNS), such as neutrophils and astrocytes, to repair the wound (Fawcett et al., 2012). In this way, the glial scar is formed and difficult to be removed (O'Shea et al., 2017).

Mesenchymal stem cells (MSCs), a population of heterogeneous stem cells, can differentiate into other tissue cells or promote tissue recovery through paracrine neurotrophic factors or anti-inflammatory factors in curing diseases, such as Parkinson's disease, Alzheimer's disease and ocular hypertension (Uccelli et al., 2008; Kang et al., 2016; Ji et al., 2018; Vilaça-Faria et al., 2019). TNF-stimulated gene 6 (TSG-6) is a 30-kDa glycoprotein, anti-inflammatory cytokine, secreted by human umbilical cord mesenchymal stem cells (hucMSCs) in in curing acute lung injury and peritonitis lung injury and peritonitis (Danchuk et al., 2011; Wang et al., 2018). It is an enzyme that catalyzes the covalent modification of non-sulfated Glycosaminoglycans (GAG) hyaluronic acid (HA). It has a special function: binding GAG and proteoglycans to remodel the extracellular matrix (Milner and Day, 2003; Day and Milner, 2019). Roura et al. reported that multipotent mesenchymal stromal cells could secrete TSG-6 through extracellular vesicles, which regulate inflammation, and have a therapeutic effect on brain injury (Roura et al., 2020). Coulson-Thomas et al. found that TSG-6 could participate in the formation of glial scars and have anti-inflammatory effects (Coulson-Thomas et al., 2016). In addition, TSG-6 can also repair pulmonary fibrosis, caused by Covid-19, by inhibiting the proliferation of inflammatory T cells, regulating the secretion of inflammatory factors such as TGF-β and IFN-γ (Srour and Thébaud, 2015; Monguió-Tortajada et al., 2020).

Hence, we speculated that hucMSC could have tissue repairing and anti-inflammatory effects to SCI via TSG-6 paracrine. We hope this study can provide a theoretical basis for hucMSCs targeted therapy in the future.

Materials and Methods

Animals

We used 66 female Sprague Dawley (SD) rats (Hunan Slack Jingda Experimental Animal Co., Ltd, Changsha, China) at six weeks old, 200-250 g weight in this experiment. Animals were raised in Department of Laboratory Animal Science (Central South University, Changsha, Hunan, China), and were housed in separate cages (SPF+IVC) with free food, water and 12 h light-dark cycle for seven days before the experiment. The animal studies were all conducted according to the "Guide for the Care and Use of Laboratory Animals, 8th ed., 2010" (National Institutes of Health, Bethesda, MD) and were approved by the Institutional Animal Care and Use Committee of Central South University (Changsha, China; Permit Number: 2019sydw0124).

HucMSCs Culture

We collected hucMSCs from the Hui Yisen Cell Gene Engineering Company (Changsha, Hunan, China). The stem cells were cultured using F12-DMEM (DMEM, Gibco, Cat# 11320082, USA) which containing 10% FBS (FBS, Gibco, Cat# 16140071, USA) for the primary culture (37°C, 5% CO_2) and were passaged after reaching 80%-90% confluence. P5 cells were used in all experiments and counted by using a hemocytometer. Flow cytometry was used to detect surface antigen of hucMSCs. The antibodies (CD34, CD45, CD73, CD90, CD105, HLA-DR) were contained in Human MSC Analysis Kit (BD Biosciences Cat# 562245, RRID: AB_2869404, USA). CD44 (BD Biosciences, Cat# 555478, RRID: AB_395870, USA), CD29 (BD Biosciences, Cat# 555443, RRID: AB_395836, USA) and CD31 (BD Horizon, Cat# 564630, USA) were also used in flow cytometry. When hucMSCs reaching 80%-90% confluence, we inoculated hucMSCs in a six-well plate. After 24 h, we used Mesenchymal Stem Cell Osteogenic Differentiation Kit (Trevigen, Cat# 5011-024-K, USA) to induce osteogenic differentiation of hucMSCs cells. Then, after calcium nodules were formed and stained it with Alizarin Red. We also used Mesenchymal Stem Cell Adipogenic Differentaition Kit (Trevigen Cat# 5010-024-K, USA) to induce adipogenic differentiation of hucMSCs cells and stained with oil red O.

Transfecting hucMSCs With TSG-6 siRNA

P5 cells were plated into the 6-well plates. Until density reached 70%-90%, hucMSCs were transfected with 20 nM siRNA against human TSG-6 (Santa Cruz Biotechnology Cat# sc-39819, USA) or NC siRNA (Santa Cruz Biotechnology Cat# sc-37007, USA). While transfecting, we diluted TSG-6 siRNA with Lipofectamine 3000 (Thermo Fisher Scientific Cat# L3000001, USA) in Opti-MEM (Gibco Cat# 31985062, USA), and cultivated transfected cells with Opti-MEM for 6 h. After 6 h, the Opti-MEM were changed to F12-DMEM medium (containing 10% FBS), and cultured cells for 48 h. After 48 h, western blot was used to detect the expression of TSG-6 in hucMSCs.

Experimental Animal Model

Rats were anaesthetized with intraperitoneal injection of pentobarbital (ZaoZhuang Water Tailan Chemical Co. Ltd., Cat# No. 57-33-0, China) (about 40 mg/kg). Then we used laminectomy to peel off the lamina at T10. After the spinal cord was exposed, we clipped the T10 spinal

cord with an artery clamp for 2 min. The SCI model was successfully created when the hind limbs and tail twitched.

Rats were randomly divided into five groups:

In Sham groups, rats were stripped the lamina did not damage the spinal cord (n = 18);

In SCI + PBS (PBS) groups, the SCI rats were injected with phosphate-buffered saline (PBS), 24 h after SCI (n = 18);

In SCI + hucMSCs groups, the SCI rats were injected with $5*10^6$ hucMSCs groups, 24 h after SCI (n = 6);

In NC hucMSCs groups, the SCI rats were injected with $5*10^6$ pre-treated negative control siRNA hucMSCs groups, 24 h after SCI (n = 12);

In TSG-6-KD hucMSCs groups, the SCI rats were injected with $5*10^6$ pre-treated TSG-6 siRNA hucMSCs (n = 12), 24 h after SCI.

After the operation, the SCI rats were urinated artificially twice a day. At 48 h after injection of hucMSCs, all the rats were euthanized, and the spinal cords were taken out (Figure 1).

Protein Extraction and Western Blot (WB)

The spinal cords on T10 were collected (n = 6) and extracted protein by RIPA (CWBIO, Cat# CW2333S, China) and protease inhibitor (CWBIO, Cat# CW2200S, China). We used BCA Kit to (Thermo Scientific, Cat# 23225, USA) determine protein concentration. Then, we used western blot to detect the expression of target proteins. After electrophoresis, the protein was transferred to PVDF membrane (Millipore, Billerica, MA, USA), and sealed with 5% skimmed milk for 2 h. The protein strips were incubated with primary antibody overnight 4°C. Relative primary antibodies were rats anti-TSG-6 (1:800, Santa Cruz Biotechnology, Cat# sc-65886, RRID: AB_1130443, USA), rats anti-MBP (1:1000, R&D, Cat# MAB42282, USA), rats anti-GAPDH (1:4000, Proteintech, Cat# 10494-1-AP, RRID: AB_2263076, USA). Finally, the strips were incubated with suitable secondary antibody, 1 h at room temperature. Protein bands were detected by Enhanced Chemiluminescence Kit (ECL; Thermo Scientific Pierce, Cat# A38554, USA). The protein bands were quantitated by ImageJ software (National Institutes of Health, USA).

Morphology Staining

Spinal cords centered on T10 (n = 6) were taken out (1 cm tissue segments) and fixed for 24 h in paraformaldehyde solution (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) (4% in 0.1 M PBS (Solarbio, Cat# P1010, Beijing, China) at 4°C). Then, tissues were dehydrated by graded ethanol. Finally, the tissues were embedded in OTC (Sakura Finetek, USA), and cut into 15-um-thick sections by slicer (Leica, Wetzlar, Germany). Tissue sections were stained with Hematoxylin-Eosin (HE) staining Kit (Beyotime, Cat# C0105S, Shanghai, China) and the Nissl Staining Solution (Meilunbio, Dalian, Cat# MA0129, Liaoning, China). The images were captured on Nikon confocal microscope (Nikon Instruments, Inc., Japan).

Immunohistochemistry and Immunofluorescence

For immunohistochemistry staining, the sections were incubated in 0.3% hydrogen peroxide/PBST (1 L PBS + 1 mL TritonX-100 (Solarbio, Cat# T8200, Beijing, China)) for 30 min. Then, the sections were blocked with normal horse serum (Beyotime, Cat# C0262, Shanghai, China)/PBST (1:200) for 2 h. Then they were incubated with anti-Ly-6G antibody (1:500, Biolegend, Cat# RB6-8C5, RRID: AB_313368, USA), overnight at 4°C. Then slices were incubated with pan secondary antibody (Vectorlabs, Cat# BA-1300-2.2, USA), at 37°C for 2 h. At last, the slices were washed

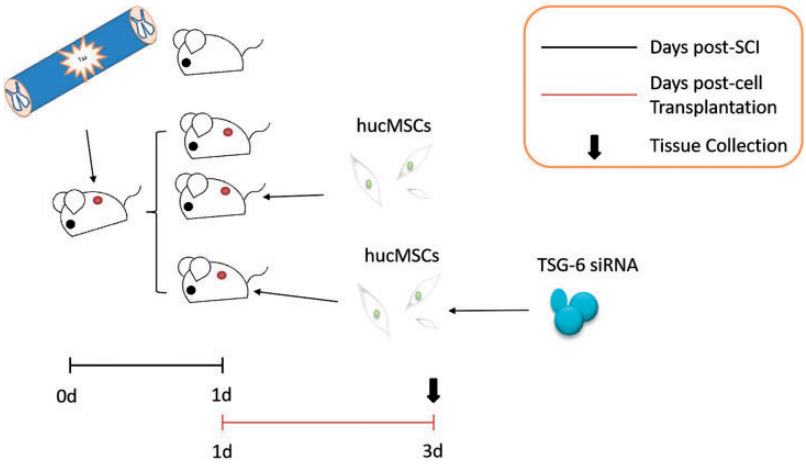

Figure 1. Experimental Scheme for hucMSCs Transplantation in SCI Rats.

with PBS and sealed with the coverslip by neutral resin (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China).

For immunofluorescence staining, the sections were blocked in normal donkey serum/PBST (1:200) for 2 h. Then they were incubated with rats anti-MBP antibody (1:1000, R&D, Cat# MAB42282, USA), rebbits anti-GFAP antibody (1:1000, Novus, Cat# NB300-141, RRID: AB_10001722, USA) or rats anti-TSG-6 antibody (1:250, Santa Cruz Biotechnology), at 37°C for 1 h and washed with PBS. Then sections were incubated with fluorescence secondary antibody (Alexa Fluor 488 (1:400, Servicebio, Cat# GB25303, China); Alexa Fluor Cy3 (1:800, Servicebio, Cat# GB21302, China)). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Images were captured by Nikon confocal microscope (Nikon Instruments, Inc., Japan). At last, the slices were sealed with the coverslip by glycerin (Solarbio, Beijing, China).

Statistical Analysis

The data were expressed as Mean ± SD. Prism Graph Pad (version 7.0, La Jolla, CA) was used to perform the statistical analysis. One-way ANOVA and Tukey's test (analysis of variance) were used to verify the differences between groups. $p < 0.05$ was considered statistically significant.

Results

Characterization of hucMSCs

We got P3 hucMSCs from Hui Yisen Cell Gene Engineering Company (Changsha, Hunan, China), and it exhibited a flattened and spindle shape (Figure 2A). In vitro differentiation model, hucMSCs successfully differentiated into osteoblasts or adipocytes under the induction of special induction medium (Figure 2B and C). In flow cytometry results, the hucMSCs showed positive for CD29 (99.87%), CD44 (99.31%), CD73 (94.41%), CD90 (99.17%), and CD105 (99.17%), and negative for CD34 (1.64%), CD45 (0.22%), CD31 (0.23%) and HLA-DR (0%), $p < 0.05$ (Figure 2D). These positive antigens are all specific antigens of hucMSCs.

HucMSCs Could Promote the Expression of TSG-6 in SCI Rats

SCI can cause severe inflammation. Excessive inflammation can hinder tissue repair and neuron regeneration. TSG-6 is an anti-inflammatory factor, which can reduce inflammation after injury. Therefore, we tested whether hucMSCs treatment may increase the expression of TSG-6 in SCI rats. In our results, the level of TSG-6 in

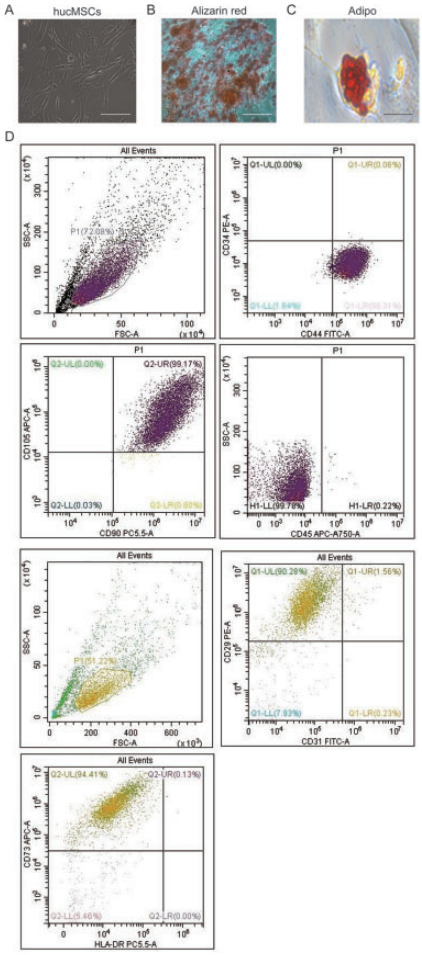

Figure 2. Typical Characteristics of hucMSCs. A. hucMSCs morphology was spindle-shaped (bar = 50 μm). B. hucMSCs' osteogenesis (bar = 200 μm) and adipogenic differentiation (bar = 25 μm). C. hucMSCs' expression of CD44, CD90, CD105, CD29, CD73 was positive. However, CD34, CD45, CD31 and HLA-DR was negative; n = 6.

SCI + hucMSCs group was higher than the SCI + PBS group (Figure 3). So, hucMSCs could promote the secretion of TSG-6 in SCI rats comparing to the PBS groups.

TSG-6 Could Alleviate the Inflammation After SCI

We found that the hucMSCs could promote the secretion of TSG-6 in vivo (Figure 3). Although, we speculated that the up-regulation of TSG-6 was secreted by hucMSCs. We still didn't know the effects of TSG-6 in vivo. Hence, we used siRNA to knock out the expression of TSG-6 in hucMSCs (Figure 4A and B). We used TSG-6-KD hucMSCs to treat SCI rats, and compared its therapeutic effect with NC hucMSCs group. Since TSG-6 is an anti-inflammatory cytokine. First, we tested whether it could regulate neutrophils. A large number of neutrophils could accumulate in the injured area. Although the infection can be reduced, excessive neutrophil

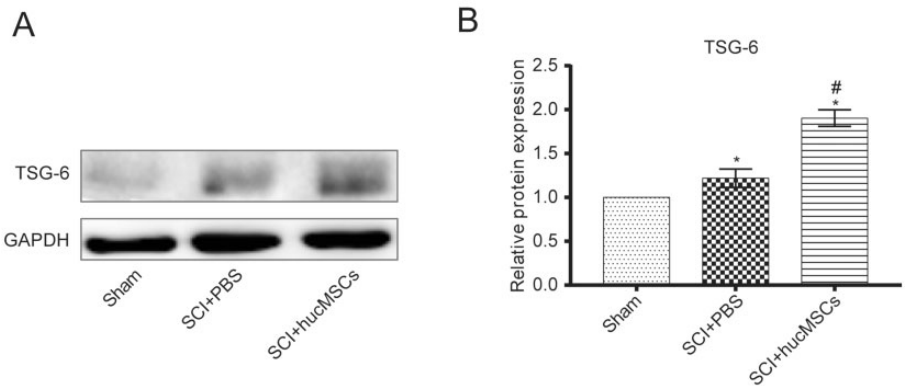

Figure 3. The Expression of TSG-6 After SCI. A. The protein levels of TSG-6 were determined by WB. B. Quantitative analysis of the protein levels of TSG-6. The results were expressed as the Mean ± SD, *$p < 0.05$, verses Sham groups. #$p < 0.05$, verses SCI+PBS groups. n = 6.

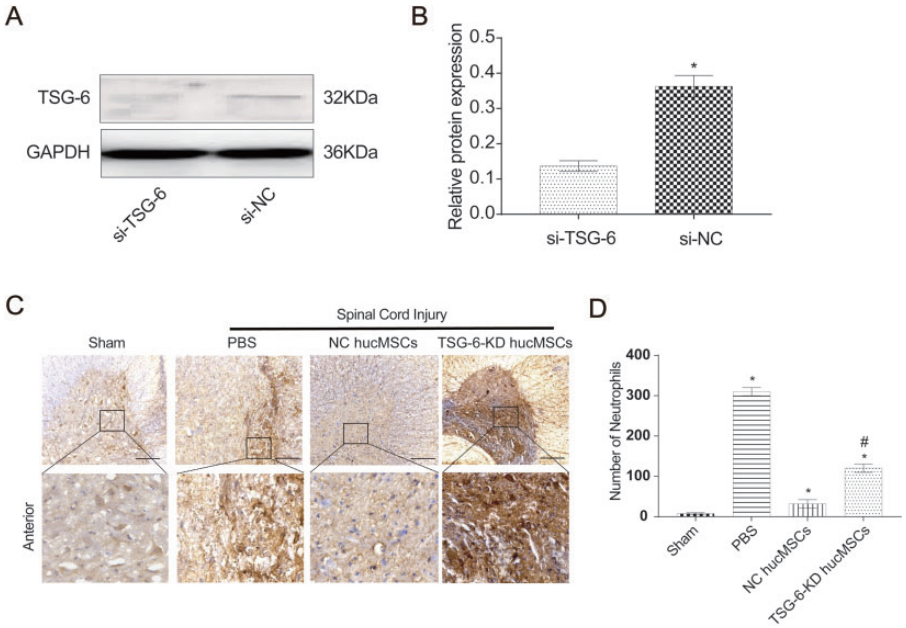

Figure 4. TSG-6 Alleviated the Inflammation by Decreasing Neutrophils Infiltration After SCI. A. The protein level of TSG-6 was determined by western blotting. B. Quantitative analysis of the protein level of TSG-6 using ImageJ. C. Immunolabelling of the neutrophils protein, 72 h after the SCI in four groups: Sham, PBS, NC hucMSCs and TSG-6-KD hucMSCs. Bar = 200 μm. Enlarged pictures after the black frame (50 μm). D. Quantitative analysis of the protein levels of neutrophils by ImageJ, the results were expressed as the Mean ± SD, *$p < 0.05$, verses Sham groups. #$p < 0.05$, verses NC hucMSCs groups, n = 6.

accumulation is not suitable for tissue recovery . Therefore, we examined the infiltration of neutrophils after SCI by immunohistological staining. In TSG-6-KD hucMSCs groups, we found that neutrophil aggravated inflammation like in PBS group (Figure 4C and D). However, the NC hucMSCs groups kept this suppressing inflammation skill. Thus, our results demonstrated that hucMSCs paracrine TSG-6 could alleviate the inflammation after SCI.

TSG-6 Could Reserve Demyelination and Inhibit Activation of Astrocyte After SCI

SCI could cause the activation of astrocytes and myelin sheath loss in the CNS. Hence, we tested the expression of the astrocyte (GFAP) which is the component of glial scar [23], and myelin basic protein (MBP) which is the basic component of myelin sheath [24]. Double labeling of TSG-6 and GFAP could prove whether TSG-6 acts on

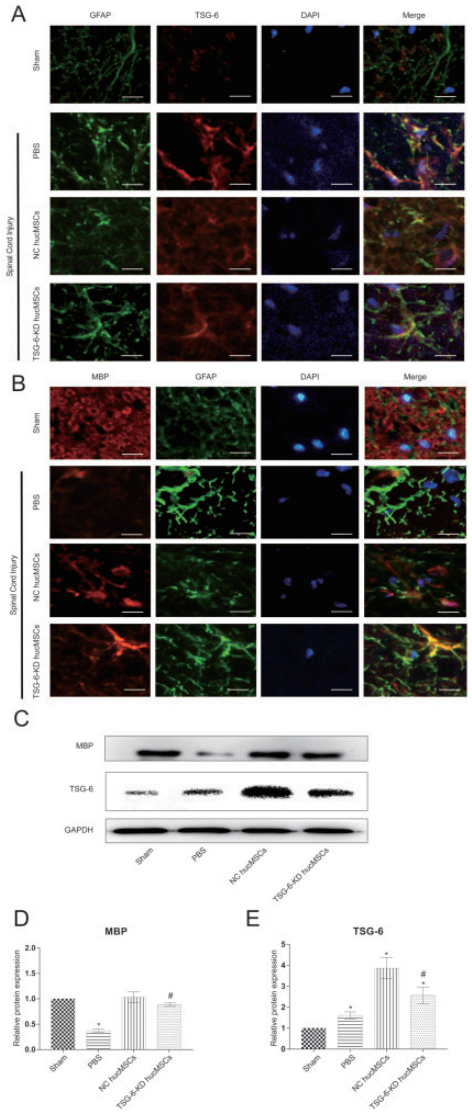

Figure 5. TSG-6 Reserved Myelin Sheath and Inhibiting Neuroinflammation After SCI. A. Immunofluorescence double labelling of the TSG-6 and GFAP, 72 h after the SCI in four groups: Sham, PBS, NC hucMSCs and TSG-6-KD hucMSCs. B. Immunofluorescence double labelling of the GFAP and MBP, 72 h after the SCI in four groups: Sham, PBS, NC hucMSCs and TSG-6-KD hucMSCs. Bar = 50 μm. C. The protein levels of MBP and TSG-6 were determined by western blotting. D.E. Quantitative analysis of the protein levels of MBP and TSG-6 using ImageJ. The results were expressed as the Mean ± SD, $*p < 0.05$, verses Sham groups. $\#p < 0.05$, verses NC hucMSCs groups. n = 6.

astrocytes. GFAP and MBP dual labeling could prove whether astrocytes affected by TSG-6 act on myelin. 72 h after SCI, we observed that the effect of TSG-6 on the expression of GFAP (Figure 5A) and MBP (Figure 5B). In NC hucMSCs groups, the expression of TSG-6 was higher and the expression of GFAP was lower comparing to the TSG-6-KD hucMSCs groups, suggesting that paracrine TSG-6 of hucMSC could decrease the expression of astrocytes after SCI (Figure 5A). The expression of MBP was higher in NC hucMSCs groups comparing to the TSG-6-KD hucMSCs groups, which suggested that paracrine TSG-6 of hucMSC could alleviate demyelination after SCI (Figure 5B). WB results also shown the same result (Figure 5C). After we silenced the expression of TSG-6 in hucMSCs, the level of TSG-6 and MBP reduced comparing to the NC hucMSCs groups, in vivo (Figure 5C and D). Thus, these results suggested that TSG-6 could decrease demyelination and the active of astrocytes.

Tsg-6 Could Promote Tissue Repair and Neuron Survival After SCI

72 h after of SCI, HE staining (Figure 6A) and Nissl staining (Figure 6B) were performed to identify changes of spinal cord. In the HE staining, compared with the Sham groups, the PBS groups had obvious bleeding and the neurons lost normal shape and there were abnormal boundaries of tissue proliferation around the injured neurons. In the NC hucMSCs groups, the number of neurons increased, and the bleeding decreased. In the TSG-6-KD hucMSC groups, although there was no obvious bleeding, the number of neurons were less than the NC hucMSCs groups.

In the Nissl staining, the Nissl bodies in the Sham groups showed normal shape (tabby-like) and numbers. In the SCI groups, the number of Nissl bodies decreased, and the color became lighter. Compared with the NC hucMSCs groups, the number of the Nissl bodies in the PBS groups were significantly reduced (Figure 6C). Although the TSG-6-KD hucMSCs groups had more numbers than the PBS groups, their Nissl bodies were smaller and blur edges and different shape.

Discussion

The purpose of this study was to test whether hucMSCs' paracrine TSG-6 has therapeutic effects on SCI. After we created SCI models, we injected them with Control siRNA treated hucMSCs or TSG-6 siRNA treated hucMSCs, respectively (Figure 1). Our results showed TSG-6 could promote tissue repair, decrease the loss of the number of neurons and reduce the infiltration of immune cells after SCI. Moreover, TSG-6 could reduce the expression of astrocytes and increase the expression of myelin basic protein, suggesting that the TSG-6 could reduce neuroinflammation and demyelination.

According to report, hucMSCs have therapeutic effects on curing SCI by reducing the inflammation (Zhang et al., 2013; Wu et al., 2020). As described in previous studies, hucMSCs' paracrine exosomes and anti-inflammatory factor promoted tissue recovery and improved locomotor function (Chen et al., 2016). For

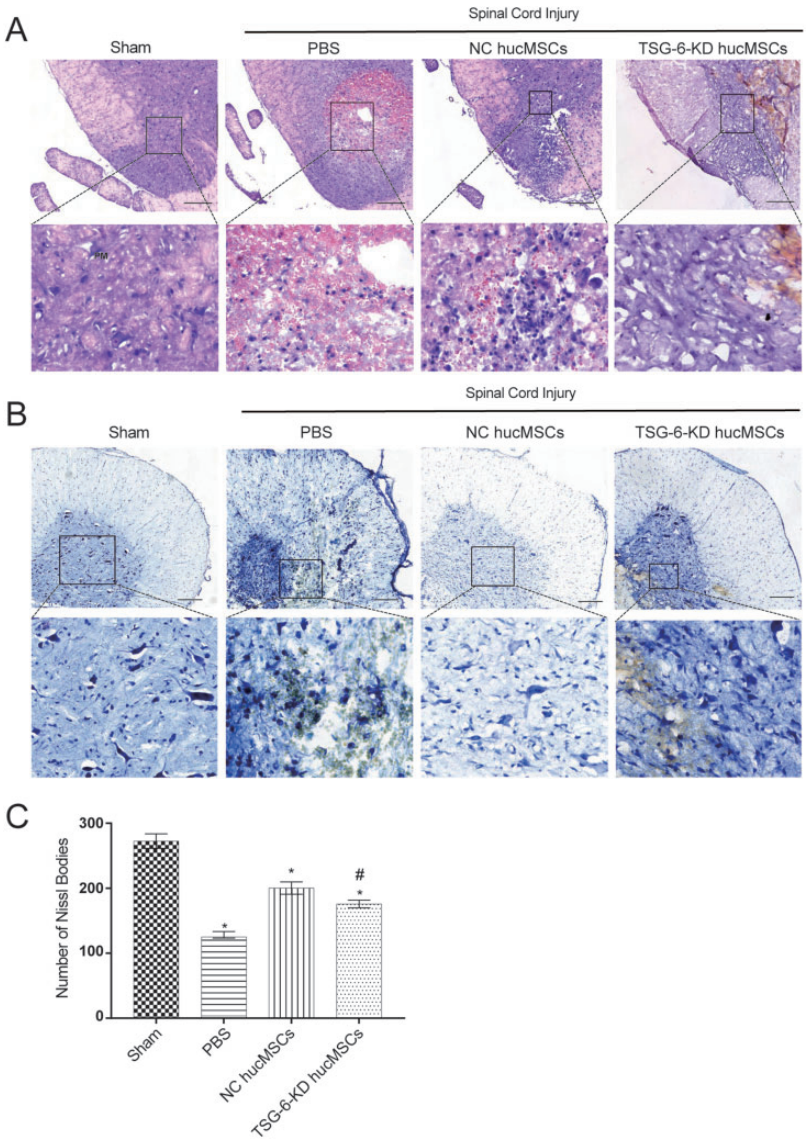

Figure 6. TSG-6 Could Promote Tissue Repair and Neuron Survival After SCI. A. HE staining of 72 h after SCI rats in four groups: Sham, PBS, NC hucMSCs and TSG-6-KD hucMSCs. B. Nissl staining of 72 h after SCI rats in four groups: Sham, PBS, NC hucMSCs and TSG-6-KD hucMSCs. Bar = 200 μm. Enlarged pictures after the black frame. Bar = 50 μm. C. Quantitative analysis of the number of neurons using ImageJ, the results are expressed as the Mean ± SD, $*p < 0.05$, verses Sham groups, $\#p < 0.05$, verses NC hucMSCs groups, n = 6.

instance, Chaubey et al. (2018) have shown that the exosomes of hucMSCs contain TSG-6, which could reduce lung inflammation with bronchopulmonary dysplasia, reduce brain cell death and hypomyelination reversed in newborn mice. More recently, An et al. (2020) have also reported how TSG-6 within extracellular vesicles derived from mesenchymal stem/stromal cell were essential in modulating exacerbated inflammation to ensure colitis tissue regeneration and repair. In our research, TSG-6 reduced neuronal damage (Figure 3) and inflammatory cell infiltration (Figure 4) at the injured site. Our results are consistent with their conclusions. Therefore, we speculate that TSG-6, which has therapeutic effects on SCI, may be secreted from hucMSCs exosomes. We will continue to confirm this view in the future studies.

The etiology of CNS disease is partly due to astrocyte dysfunction and activation. Our results suggested that the TSG-6 reduced the expression of astrocytes (GFAP) and increased the expression of myelin basic protein (MBP) (Figure 5). We noticed that, in TSG-6-KD hucMSCs groups, the expression of MBP were significant difference comparing with NC hucMSCs groups, but it was not a huge difference. We speculated that 72 h was still in an early stage after SCI, and the degeneration of myelin sheath was not very obvious. We would test more time points after SCI in the future. Coulson-Thomas only

discussed the possibility of TSG-6 involved in the formation of glial scars after SCI but did not confirm it with specific treatments (Coulson-Thomas et al., 2016). GFAP is one of the components of glial scars, we may speculate that TSG-6 may be involved in the formation of glial scars. Also, we would focus on the types of astrocytes. Neuroinflammation would turn astrocytes into two active types, pro-inflammatory type and anti-inflammatory type (Liddelow and Barres, 2017). We need to find out whether TSG-6 could regulate the activation of astrocytes (Zamanian et al., 2012; Lian et al., 2015). These still need our further verification.

In our previous work, we found out, in rats' model of inflammatory pain, acetylated histone three may be involved, the expression of acetylated histone three significantly upregulated in the spinal cord dorsal horn neurons and glial cells, especially in astrocytes (Li et al., 2012). In this way, since we found TSG-6 could attenuate astrocytes' expression. We will further explore if TSG-6 could regulate acetylated histone 3 reduce nerve pain after SCI.

Our experiment still has some shortcomings. Although we have demonstrated the therapeutic effects of TSG-6 on SCI, we have not proven these effects were achieved through which specific signaling pathways. Indoleamine-2,3-dioxygenase (IDO), the upstream molecule of TSG-6, was activated by immune response in acute lung injury (Wang et al., 2018) and it suppressed T cells by metabolic depletion of tryptophan (Munn and Mellor, 2013). Our future research will focus on the specific signaling pathway of TSG-6.

Conclusion

Our results suggested that hucMSCs paracrine TSG-6 could reduce immune cells infiltration and demyelination in early-stage after SCI. It could decrease neuron loss and promote tissue recovery.

Highlights

hucMSCs paracrine TSG-6 reduces immune cells infiltration after SCI.

hucMSCs paracrine TSG-6 recovers myelin sheath after SCI.

hucMSCs paracrine TSG-6 decreases neuron loss, promotes tissue recovery.

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Author Contributions

Ziling Liao contributed to the concept and design, performed the research, acquisition of data or analysis and drafted the article and revised it. Wei Wang, Weiyue Deng, Yuying Zhang, Aishi Song and SiHao Deng performed the research, analyzed the data, revised the article. BinNi, Yiping You, Huifang Zhao, Shusheng Zhang and Zhiyuan Li helped performing the analysis with constructive discussions. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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