

Adipose mesenchymal stem cell transplantation alleviates spinal cord injury-induced neuroinflammation partly by suppressing the Jagged1/Notch pathway

Zhou Zhilai¹†, Tian Xiaobo^{2,1}†, Mo Biling³, Xu Huali⁴, , Yao Shun¹, Huang Zixiang¹, Huang Lishan¹, Xie Huan¹, Xu Liwei^{2,1}, Zhang Hui^{1,2*}

1. The Spine Department, Orthopaedic center, Guangdong Second Provincial General Hospital, Guangzhou, China

2. The Second School of Clinical Medicine, Southern Medical University, Guangzhou, China

3. Department of Cardiology, The Liwan Hospital of the Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, China

4. Department of Anesthesiology, Zhu Jiang Hospital, Southern Medical University, Guangzhou, China

* Correspondence: 273344078@qq.com, zhouzhilainba@163.com

The Spine Department, Orthopaedic center, Guangdong Second Provincial General Hospital, Guangzhou, China

†Zhou Zhilai and Tian Xiaobo contributed equally to this work.

Background

The therapeutic effects of adipose-derived mesenchymal stem cell (ADSC) transplantation have been demonstrated in several models of central nervous system (CNS) injury and are thought to involve the modulation of the inflammatory response. However, the exact underlying molecular mechanism is poorly understood. Activation of the Jagged1/Notch signaling pathway is thought to involve inflammatory and gliotic events in the CNS. Here, we elucidated the effect of ADSC transplantation on the inflammatory reaction after spinal cord injury (SCI) and the potential mechanism mediated by Jagged1/Notch signaling pathway suppression.

Methods

Using a mouse model of compression SCI, ADSCs and Jagged1 small interfering RNA (siRNA) were injected into the spinal cord. Locomotor function, spinal cord tissue morphology and the levels of various proteins and transcripts were compared between groups.

Results

ADSC treatment resulted in significant downregulation of proinflammatory mediator expression and reduced ionized calcium binding adapter molecule 1 (Iba1) and ED1 staining in the injured spinal cord, promoting the survival of neurons. These changes were

accompanied by improved functional recovery. The augmentation of the Jagged1/Notch signaling pathway after SCI was suppressed by ADSC transplantation. The inhibition of the Jagged1/Notch signaling pathway by Jagged1 siRNA resulted in a decrease in SCI-induced proinflammatory cytokines as well as the activation of microglia. Furthermore, Jagged1 knockdown suppressed the phosphorylation of JAK/STAT3 following SCI.

Conclusion

The results of this study demonstrated that the therapeutic effects of ADSCs in SCI mice were partly due to Jagged1/notch signaling pathway inhibition and a subsequent reduction in JAK/STAT3 phosphorylation.

Keywords: Adipose, mesenchymal stem cell, spinal cord injury, neuroinflammation, Jagged1, Notch.

Introduction

Spinal cord injury (SCI) is one of the most challenging clinical issues, with an incidence of 15-40 cases per million people worldwide every year[1]. SCI induces a cascade of secondary tissue damage that limits spontaneous neural tissue regeneration, often leading to severe and permanent paralysis, despite great efforts; however, at present, SCI repair remains a major therapeutic challenge for researchers[2]. Recent advances in SCI research have indicated that the neuroinflammatory process caused by injuries to the central nervous system (CNS), including the infiltration of macrophages into the injured tissue and the secretion of

inflammatory cytokines, is one of the major causes of mortality and unfavorable outcomes in SCI patients.[3]

With the development of stem cell technology, the immunomodulating function of stem cell transplantation, especially that of mesenchymal stem cells (MSCs), also known as mesenchymal stromal cells, has become a hot topic for the treatment of SCI. MSCs were originally isolated from bone marrow, and similar populations have been isolated from other tissues[4]. Our previous study demonstrated that adipose tissue-derived mesenchymal stem cells (ADSCs) are a superior alternative to MSCs from other tissues because of their abundant availability and excellent expansion and proliferative capacities[5]. MSCs have widely been used in experimental and clinical settings and have exhibited tangible therapeutic potential in various central nervous system (CNS) conditions, such as ischemic stroke[6], cerebral ischemia[7], multiple sclerosis[8] and SCI[9]. It has been reported that MSC transplantation can reduce the inflammatory reaction in the pathological process.[10] Our previous study also demonstrated that ADSCs can alleviate the infiltration of ED1+ macrophages and suppress the inflammatory response after SCI[11]. However, the precise mechanism underlying the effects of transplanted MSCs on the inflammatory reaction following SCI is still unclear.

The Notch1 pathway is a highly conserved signaling system that is critical for cell fate decisions, tissue morphogenesis and many cellular processes[12]. The interaction of Notch receptors with

their ligands (Delta1 or Jagged1) leads to the proteolytic cleavage of the transmembrane Notch receptor, giving rise to the release of the Notch intracellular domain (NICD) from the membrane, after which the NICD migrates into the nucleus. In the nucleus, NICD interacts with the transcription factor RBP-Jk, which promotes the expression of transcriptional activators and thereby induces the expression of target genes, mainly hairy and enhancer of split (Hes) isoforms 1 and 5[13]. Signal transducer and activator of transcription 3 (Stat3) is a critical regulator of astrogliosis and scar formation after CNS injury. The activation of Stat3 via phosphorylation by Janus kinases (JAKs) has been demonstrated in a variety of disease models, including models of Alzheimer's and Huntington's diseases[14], cerebral ischemia[15] and SCI[16, 17]. Several studies have demonstrated a strong association between Notch and JAK/STAT3 signaling, and a change in Notch expression can alter Stat3 phosphorylation and activity[18, 19]. Recent studies have reported that during inflammatory and gliotic events in the CNS, Jagged1/Notch signaling sustains inflammation in part through the JAK/STAT3 signaling pathway[20]. MSC transplantation has been demonstrated to suppress Notch1 signaling in various disease models, including models of subarachnoid hemorrhage[21], inflammatory bowel disease[22], and lupus[23]. However, whether MSC administration inhibits the Notch1 signaling pathway in SCI is still unclear.

In this study, we investigated the spatiotemporal expression of

Notch signaling in a mouse model of SCI and then elucidated the signaling pathways underlying the functional recovery of SCI mice treated with ADSC transplantation.

Materials and Methods

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Guangdong Second Provincial General Hospital, Guangzhou, China.

Animals and experimental groups

The experiments were performed as described below.

Part 1 To evaluate the effects of ADSC treatment on the recovery of SCI mice and the activity of Notch1 signaling after SCI, a total of 114 8- to 12-week-old female C57Bl/6 mice were divided into three experimental conditions: (i) The sham group underwent laminectomy without SCI, (ii) the control group underwent SCI followed by injection of PBS without cells and (iii) the ADSCs group underwent SCI followed by transplantation of ADSCs. The number of mice used in each experimental group is listed in Table 1.

Table 1. Experimental groups.

Survival time		Analysis	
Animal groups			
Control	ADSCs	Sham	
3 days	NICD/Jagged1/RBP-JK WB	4	4

4			
	NeuN immunoreactivity	4	4
4			
7 days	IL-6/TNF- α /IL-1 β Q-PCR	4	4
4			
	GFAP/Jagged1/NICD/notch1/ NeuN/Iba-/ED1		
	and activated caspase-3 immunoreactivity	10	10
10			
	NICD WB	4	4
4			
14 days	NICD WB	4	
4	4		
	NeuN immunoreactivity	4	4
4			
21 days	NICD WB	4	4
4			
28 days	NeuN immunoreactivity	4	
4	4		

The numbers of mice in each group used in this part of the experiment is provided.

Part 2

To validate the hypothesis that the Jagged1/Notch signal pathway participates in the protective mechanism of SCI, a lentiviral vector

carrying Jagged1-siRNA (Shanghai Lianfeng Biotechnology Co., Ltd. China) was injected into the spinal cord 72 hours before SCI modeling to inhibit the Jagged1/Notch pathway. A total of 48 female C57Bl/6 mice were divided into four experimental conditions: (i) the sham group, (ii) the SCI+PBS-injected (control) group and (iii) the SCI+Jagged1 siRNA-injected group, which received an injection of 2.5 μ l of lentiviral vector (4×10^8 IU/ml) 3 days before SCI, (iv) and the SCI+Scramble siRNA-injected group, which were used as controls for the Jagged1 siRNA-injected group and received an injection of scramble lentiviral vector. The number of mice used in each experimental group is listed in Table 2.

Table 2. Experimental groups.

Survival time	Analysis	Animal groups	
		Sham	Control
SCI+	SCI+		
	Scramble siRNA		
	Jagged1 siRNA		
3 days	NICD/Jagged1/RBP-JK	4	4
4			
	and JAK/STAT3/p-STAT3 WB		
	IL-6/TNF- α /IL-1 β Q-PCR	4	4
4			

The number of mice in each group used in this part of the experiment is provided.

Preparation of ADSCs

ADSCs were isolated as previously described[11]. Adipose tissues from the inguinal pads were dissected, and upon the removal of debris, the tissues were then enzymatically dissociated with 0.1% collagenase type I (Sigma, St. Louis, MO) at 37°C for 60 min under shaking. The digested tissue was centrifuged at 1,500 rpm for 5 min and resuspended in saline for a total of 2 times. Isolated cells were resuspended in Dulbecco's modified Eagle medium (DMEM; Gibco) with 10% fetal bovine serum (Gibco) and maintained at 37°C in 5% CO₂. Following overnight incubation, the flasks were washed extensively with PBS to remove nonadherent cells. Fluorescence-activated cell sorting analysis was used to identify the phenotype of the cells. CD29, CD44, CD90 and CD45 were detected to confirm the MSC identity of the cells. ADSCs at passage 3 were used in this study.

SCI model and ADSC transplantation

After anesthetization by inhalation of 1.5% isoflurane, the animals underwent dorsal laminectomy at the tenth thoracic vertebral level (T10), and the spinal cord and dura mater were exposed. A moderate contusion injury (~ 50 kdyn) of the spinal cord was produced using the Infinite Horizon Impactor (Precision Systems and Instrumentation) as previously described[24]. After injury, depending on the experimental group, 3 µl of saline or ADSCs (1×10^6 cells) was injected directly into the SCI epicenter with a Hamilton syringe positioned with a stereotaxic instrument

(Kopf Instruments, Tujunga, CA, USA) at a flow rate of 0.4 $\mu\text{l}/\text{min}$. The overlying muscle layers were then sutured, and the cutaneous layers were stapled. The bladders of the mice were manually and gently expressed to prevent urinary tract infections until the reflexive control of micturition was restored. Depending on the experiment performed, SCI mice were killed by transcardial perfusion 1, 4, 7, 14, and 28 days postcontusion.

Behavioral analyses

Functional recovery after SCI was assessed with Basso Mouse Scale (BMS) scores[25]. Mice were tested on postoperative days 1, 7, 14, 21 and 28 for the duration of the experiments. All animals were placed in an open field and simultaneously observed by two examiners who were blinded to the identity of the animals.

Western blot analysis

On days 1, 3 and 7 after injury, mice (n=4) were transcardially perfused with ice-cold PBS (0.1 M, pH 7.4), and approximately 5 mm of each spinal cord segment, including the injury site, was collected and stored at -80°C until further analysis. Equal amounts of protein (50 μg) from different samples were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The blots were incubated with the following primary antibodies: Notch1 (Santa Cruz Biotechnology, rabbit, 1:1000), RBP-JK (Santa Cruz Biotechnology, rabbit, 1:1000), NICD (Abcam, rabbit, 1:2000), JAK (Cell Signaling, rabbit, 1:2000), p-STAT3 (Cell Signaling, rabbit, 1:2000), and STAT3 (Cell Signaling,

rabbit, 1:2000). The primary antibodies were detected with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody. β -Actin was used as an internal control (Sigma). The reactive bands were visualized using enhanced chemiluminescence reagent (Thermo Fisher Scientific, Waltham, USA), and the density of the protein bands was semiquantified using ImageJ software.

Real-time polymerase chain reaction

RNA was isolated using TRIZOL Reagent (Sigma-Aldrich), and reverse transcripts were synthesized from 1 μ g of total RNA using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara Biotechnology, Dalian, China) according to the manufacturer's instructions. Amplification was performed using the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with a two-step PCR protocol (preincubation of 10 min at 95°C followed by 30 cycles at 95°C for 15 seconds and 60°C for 1 min). The list of primer sequences is provided in Table I. After normalization to β -actin mRNA, the comparative threshold (Δ CT) method was used to examine the relative quantification of the samples (Relative Quantitation computer software; Applied Biosystems). Fold expression changes were calculated using the equation $2^{\Delta\Delta CT}$.

Table 1. Primers for real-time PCR

Gene	Sequence

IL-6

GAGTCCTTCAGAGAGATACAGAA

AC

TGGTCTTGGTCCTTAGCCAC

IL-1 β GAAATGCCACCTTTTGACAGTG

CTGGATGCTCTCATCAGGACA

TNF- α

CCCTCACACTCAGATCATCTTCT

GCTACGACGTGGGCTACAG

β -actin ATCAACGACCCCTTCATTGACC

CCAGTAGACTCCACGACATACTCA

G

Immunostaining

The mice were anesthetized and transcardially perfused with 4% paraformaldehyde in PBS on days 1, 3, 7, 14, and 28 after SCI. Spinal cords were postfixed in paraformaldehyde overnight, embedded in paraffin, sectioned in the sagittal or transverse plane at a thickness of 6 μ m, and deparaffinized and rehydrated by subsequent immersion in xylene (2 times, 10 min), 100% ethanol (5 times, 1 min), 95% ethanol (5 min), 90% ethanol (3 min), 80% ethanol (3 min), 70% ethanol (3 min), 80% ethanol (5 min) and H₂O (5 min). The following primary antibodies were used: Jagged1

(Santa Cruz, goat, 1:50), Notch1 (Santa Cruz, rabbit, 1:100), NICD (Cell Signaling, rabbit, 1:100), GFAP (Sigma-Aldrich, mouse, 1:1000; Millipore, rabbit, 1:1000), Iba1 (Abcam, Shanghai, China, rabbit, 1:1000), NeuN (Millipore, mouse, 1:1000), ED-1 (Millipore, mouse, 1:200), and activated caspase-3 (Cell Signaling, rabbit, 1:100). Antigen retrieval was performed with sodium citrate buffer (10 mM sodium citrate, pH 6, 0.01% Triton) for 20 min at 96°C in a microwave. For immunostaining with antibodies against Jagged1 (Santa Cruz, goat, 1:50) and Notch1 intracellular domain (Cell Signaling, rabbit, 1:100), mounted sections were subjected to sodium citrate buffer (10 mM sodium citrate, pH 6, in 0.01% Triton)-based antigen retrieval in a preheated pressure cooker for 5 min. The secondary antibodies (1:500) that were used were Alexa Fluor 594-conjugated goat anti-rabbit (ThermoFisher) and Alexa Fluor 488-conjugated goat anti-mouse (ThermoFisher). Finally, the tissues were counterstained with 4'6-diamidino-2-phenylindole (DAPI) and observed under a fluorescence microscope (Leica-DMI8, Leica Microsystems, Germany).

Quantitative analyses of stained tissue sections

To quantify cells of interest in immunostained sections, we obtained images of the stained sections by fluorescence microscopy (model TCS SP2; Leica Microsystems). Three slices per mouse and three fields from each slice were used for quantification; we manually outlined the cells and quantified them using ImageJ software. The threshold values were maintained at a constant level

for all analyses using ImageJ software. ED 1+, Iba 1+ and NICD+ areas were divided by the area of the image and expressed as a percentage. The number of NeuN+ cells and activated caspase 3+ cells were manually counted at 400× magnification. The average number of activated caspase 3+ cells expressing NeuN in these images was quantified.

Statistical analysis

Statistical analysis was performed using SPSS 20.0 for Windows. All data in this study are presented as the mean ± SEM. Data from more than 2 groups were analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni post-hoc testing. The differences between 2 groups were analyzed by Student's t-tests. Differences were deemed statistically significant at $P < 0.05$.

Results

ADSC administration inhibited the infiltration of macrophages and reduced the expression of inflammatory cytokines following SCI

To evaluate the effect of ADSC injection on microglial activation after SCI, the relative fluorescence intensity of the inflammatory infiltrates was analyzed 7 days after injury. In the present study, ED-1 was used as a marker of the activation of resident microglia and extravasated macrophages, while Iba 1 was used as a marker of labeled total microglia (Fig. 1A, B). We found that the ADSCs group exhibited significantly fewer areas of Iba1+ and ED 1+ cells compared to that exhibited by the control group.

Moreover, we used Q-PCR to further confirm these results. The expression levels of inflammatory markers, including TNF- α , IL-1 β , and IL-6, were significantly reduced by ADSC treatment compared to vehicle control 7 days after injury (Fig. 1C).

ADSC treatment reduced the death of neurons and promoted functional improvements after SCI

To examine whether ADSC treatment showed neuroprotective effects after SCI, we performed NeuN staining to detect surviving neurons in the ventral horn of the spinal cord on days 3, 7, 14 and 28 post-SCI. Viable neurons were defined by strong NeuN staining. Our results showed that from day 7, the preservation of neuronal integrity was significantly increased when compared to that in the control group (Fig. 2A, B). Neuronal death was confirmed by activated caspase-3/NeuN co-labeling at 7 days post-SCI. In the ventral horn of SCI mice, activated caspase-3 immunoreactivity was exhibited in $62.42 \pm 3.44\%$ of neurons, which was 1.8-fold greater than the percentage of activated caspase-3-positive neurons observed in the ventral horn of ADSCs group mice (Fig. 2C, D). We used Basso Mouse Scale (BMS) scores to evaluate functional recovery during the 4 weeks following SCI. The scores of the control group increased gradually and reached a plateau at approximately 3 weeks. Significant gains in BMS scores in SCI mice treated with ADSCs compared to PBS control group mice were found beginning on day 14 and continued until the end of the observation on day 28 ($P < 0.05$) (Fig. 2E). These data demonstrate

that ADSC transplantation can significantly improve the preservation of neuronal integrity and ameliorate the functional deficits generated in this SCI mouse model.

The Notch signaling pathway was activated in the spinal cord of SCI mice

To examine the activation of Notch signaling after SCI, we first determined the expression profile of NICD in the spinal cord of SCI mice (1, 3, 7 and 14 days) and sham mice by western blotting. We found that NICD expression in the spinal cord of SCI mice began 1 day after injury, reached a peak at 3 days and then gradually decreased to baseline at 14 days (Fig. 3A, B). Immunocytochemistry was used to further confirm the expression pattern of NICD and notch1, as shown in Figure 3C, 3 days after SCI, NICD and notch1 staining were predominantly found in the ventral horn of the spinal cord. Our results showed no obvious staining for NICD and notch1 in sham mice (data not shown). Because NICD is the activated form of Notch1, these results suggest that Notch1 signaling is activated after SCI.

Neurons and astrocytes were the cell types involved in Notch signaling in SCI mice

To confirm which cell types mediate Notch signaling in the spinal cord, we determined the cellular localization of NICD and Jagged-1 using double immunofluorescence assays. Our results showed that NICD was predominantly localized to NeuN+ neurons and that no NICD signal was observed in GFAP+ astrocytes (Fig. 4A,

C). Jagged 1 expression was clearly identifiable in GFAP+ astrocytes (Fig. 4B). There was no evidence of NICD and Jagged 1 expression in microglia (data were not shown). We found an interesting phenomenon: GFAP+ astrocytic processes (arrowheads) tended to surround NICD+ cells (arrows).

ADSC treatment inhibited the activation of the Jagged1/Notch signaling pathway

To explore the underlying mechanism of ADSC treatment in SCI mice, we performed western blotting and immunofluorescence to evaluate the expression of Notch1 signaling pathway-associated factors 3 days after ADSC treatment. The western blotting results demonstrated that SCI increased NICD, Jagged1 and RBP-JK expression and that this increase was attenuated after ADSC transplantation (Fig. 5A, C, D, E). Consistent with the results of Western blotting, IF staining showed that the intensity of NICD immunofluorescence in the center of lesion was significantly reduced after ADSC treatment (Fig. 5B).

Knockdown of the Jagged1/Notch signaling pathway by Jagged1 siRNA decreased microglial activation and inflammatory cytokine infiltration

Microglial/macrophage activation is an important process that influences post-SCI recovery. To investigate whether Jagged1/Notch influences microglial/macrophage activation after SCI, a lentivirus carrying Jagged1 siRNA was injected into the mouse spinal cord 3 days before SCI modeling to knock down

Jagged1 expression and observe the inflammatory response after SCI. The western blotting results showed that the NICD, Jagged1 and RBP-JK protein levels were significantly reduced in the mouse spinal cord 6 days after Jagged1 siRNA injection (3 days after SCI) ($P < 0.05$, Fig. 6 A, B, C, D). We next examined the effect of Jagged1 siRNA injection on activated microglia. As shown in Fig. 6 E-F, Jagged1 siRNA injection significantly decreased the number of Iba1+ cells 3 days following SCI. Q-PCR examination suggested that the SCI-induced upregulation of the proinflammatory cytokines IL-6, IL-1 β , and TNF- α was also reduced (Fig. 6G). These results suggest that Jagged1/Notch signaling sustains inflammation in the spinal cord of SCI mice.

The JAK/STAT3 pathway was repressed by the inhibition of Jagged1

Activation of the JAK/STAT3 pathway has been observed to be correlated with microglial activation in several conditions of acute injury. Several reports have shown that there is a crosstalk between the Notch pathway and the JAK/STAT3 signaling pathway. We therefore sought to determine whether the inhibition of the Jagged1/Notch1 signaling pathway can modulate JAK/STAT3 activity. Our western blotting results showed that JAK, STAT3 and phosphorylated STAT3 (markers of activation) were significantly increased 3 days following SCI but that Jagged1 knockdown significantly reduced this effect compared with that observed in the other groups (Fig. 7A-D).

Discussion

The results of the present study suggest that the administration of ADSCs immediately after contusion SCI can prevent neuroinflammation, attenuate neuronal death and eventually promote functional recovery. This neuroprotection is at least partially due to the suppression of Jagged1/Notch1 signaling, which subsequently inhibits JAK/STAT3 phosphorylation.

Traumatic injuries to the spinal cord often cause the infiltration of circulating blood cells and ensuing ischemia, edema and hemorrhage, which leads to continuous neuroinflammation and neurological deficits at the injury site and in the area adjacent to the lesion[26]. Recent progress in stem cell biology has opened up an avenue for therapeutic strategies for models of multiple CNS diseases, such as multiple sclerosis[27], TBI[28], and SCI[29] through the transplantation of stem/progenitor cells. ADSCs have been reported to ameliorate inflammation-induced CNS injury in different CNS diseases by suppressing microglial activation and inhibiting proinflammatory cytokine expression[30, 31]. Our findings are in line with these observations. In our present study, by performing immunostaining for ED-1 to label active microglia/macrophage and Iba-1 to label total microglia, we observed significant decreases in the numbers of ED-1+ and Iba 1+ cells upon treatment with ADSCs on day 7 after SCI compared to the numbers in the SCI control group. Furthermore, by Q-PCR, we found that ADSC transplantation significantly decreased the

expression of proneuroinflammatory cytokines such as IL-6, IL-1 β , and TNF- α in the injured spinal cord. These results are not surprising because activated infiltrated macroglia/macrophages are believed to secrete more detrimental and proinflammatory cytokines and factors that contribute to neural cell death after SCI[32]. In addition, astrocytes, as the most abundant cells in the CNS, undergo a dramatic transformation after injury; recent studies have identified at least two types of reactive astrocytes, A1 neurotoxic reactive astrocytes and A2 protective astrocytes, it has been shown that activated microglia promote the transformation of A2 protective astrocytes into A1 neurotoxic reactive astrocytes, which may further enhance the production of proinflammatory cytokines after SCI[33]. We next addressed whether ADSC transplantation helps maintain neuronal viability. The results of NeuN staining showed that from day 7, the number of viable, nonatrophic neurons with strong NeuN immunostaining was nearly twofold greater than that in the SCI control group. Neuronal death was also confirmed by activated caspase-3/NeuN double labeling. The percentage of neurons that displayed activated caspase-3 immunoreactivity in the mice in the SCI control group was $62.42 \pm 3.44\%$, which was 1.8-fold greater than that observed in the ADSCs group mice. These results suggest that ADSC administration reduces neuroinflammation and promotes neuronal integrity after SCI, and this conclusion was also supported by the results of functional analysis.

The precise mechanism through which MSCs affect the inflammatory reaction following SCI is still unclear. In recent years, Jagged/Notch1 signaling, which is critical for cell fate decisions and endogenous neurogenesis, has been regarded as a potential therapeutic target for modulating the inflammatory response after CNS injury[20, 34]. Preventing the cleavage of Notch1 into the Notch intracellular domain (NICD) with DAPT augments the regeneration of motor neurons in the injured spinal cord of zebrafish[35] and promotes endogenous neurogenesis and axonal reorganization in a rat stroke model[34]. Thiruma V Arumugam further demonstrated that Notch endangers neurons by modulating pathways that increase their vulnerability to apoptosis and that by activating microglial cells and stimulating the infiltration of proinflammatory leukocytes, the suppression of Notch signaling by DAPT reduces brain damage and improves functional outcomes in a mouse model of stroke[36]. These findings indicate that the activation of Notch signaling is responsible for the progression of neuroinflammation. It has been reported that MSC transplantation alleviates early brain injury in subarachnoid hemorrhage and improves osteopenia in lupus by suppressing Notch1 signaling[21, 37]. Naturally, we hypothesized that ADSC treatment may alleviate the inflammatory response and neurodegeneration by inhibiting the Jagged1/Notch signaling pathway after SCI.

In the present study, we first investigated the temporal evolution of Notch1 signaling after SCI. Because NICD is the

activated form of Notch1, we used western blotting to detect the expression level of Notch1. Our results showed that NICD was maximally activated 3 days post injury and that its expression was reversed 7 and 14 days post-SCI. This result demonstrates that Notch1 signaling was activated in the acute phase of SCI and then gradually declined in the subacute stage. To confirm which cell types of the spinal cord mediate Notch1 signaling, we determined the cellular localization of NICD1 and Jagged1 using double staining for these proteins and different neuronal and glial cell type markers. Double immunostaining showed that NICD was mainly expressed in NeuN+ neurons, whereas Jagged1 was predominantly expressed in astrocytes in the ventral horn of mice. These results suggest that Jagged1/Notch signaling was activated in these cells. Our double staining results are in agreement with several previous studies[38, 39]. Interestingly, we found that ADSC administration significantly inhibited the expression of Jagged1/Notch1 and its downstream factors, including Jagged1, NICD and RBP-JK, compared with that in the SCI control group after SCI. These results suggest that ADSC administration immediately after contusion SCI inhibits the activation of the Jagged1/Notch signaling pathway.

To evaluate whether the inhibition of Jagged1/Notch signaling reduces neuroinflammation after SCI, we injected a lentivirus carrying Jagged1 siRNA into the mouse spinal cord to knock down Jagged1/Notch. Our results show that Jagged1/Notch1 signaling

was significantly suppressed by Jagged1 siRNA at 6 days postinjection. In addition, the expression of proneuroinflammatory cytokines in the injured spinal cord was significantly reduced by Jagged1 siRNA injection 3 days post-SCI, which was confirmed by measuring the mRNA levels of IL-6, IL-1 β , and TNF- α . Taken together, these results indicate that ADSCs may alleviate the inflammatory response after SCI at least partly by inhibiting Jagged1/Notch signaling.

The Notch signaling pathway is a cell-to-cell signaling pathway that is activated by the binding of a number of different molecules to the cell surface[40]; however, our present study and previous studies have all demonstrated that the expression of notch and its downstream factors in microglia/macrophages and oligodendrocytes is negligible. The molecular mechanism by which the inhibition of the Jagged1/Notch pathway downregulates the microglia-mediated neuroinflammatory response after SCI is still unclear. A previous study suggested that during inflammatory and gliotic events in the CNS, Jagged1/Notch signaling sustains inflammation partially through the JAK/STAT signaling pathway[20]. It was previously reported that the JAK/STAT3 pathway is activated in reactive astrocytes in mouse models of Alzheimer's disease and Huntington's disease and that JAK/STAT3 knockdown significantly inhibits astrocyte reactivity, ultimately reducing the expression of Iba1+ microglia[14]. These results indicate that Jagged/Notch enhances the inflammatory response and aggravates the death of

neurons, at least in part by augmenting JAK/STAT3 phosphorylation. In the present study, we found that Jagged1 siRNA effectively knocked down the expression of Jagged1/Notch signaling and decreased microglial activation. The protein levels of JAK, STAT3 and p-STAT3 were also reduced after Jagged1 knockdown 3 days post-SCI. Based on these results, we speculate that Jagged1/Notch signaling may participate in the neuroinflammatory response in SCI by mediating the phosphorylation of the JAK/STAT3 signaling pathway.

Conclusion

Here, we enhance the understanding of ADSC-based cellular therapy by providing experimental evidence that the neuroprotective effects of ADSCs in the treatment of SCI are associated with Jagged1/Notch signaling. ADSC treatment suppresses the activation of the Jagged1/Notch pathway after SCI, which inhibits the phosphorylation of JAK/STAT3 and then alleviates the microglia/macrophage-mediated neuroinflammatory response.

Abbreviations

ADSC: Adipose mesenchymal stem cell; Hes-1: Hes family basic helix loop helix transcription factor 1; Iba1: Ionized calcium binding; JAK/STAT3: Janus tyrosine kinase/ signaltransducer and activator of transcription; SCI:spinal cord injury; CNS: central nervous system; NICD: Notch intracellular domain; GFAP: Glial fibrillary acidic protein; TNF- α : Tumor necrosis factor alpha; IL: Interleukin;

ANOVA: Analysis of variance; siRNA: Small interfering RNA; RBP-JK: recombining binding protein suppressor of hairless; BMS: Basso Mouse Scale; TBI: Traumatic brain injury.

Acknowledgements

We would like to acknowledge Luo Shiming at Fertility Preservation Lab, Reproductive Medicine Center, Guangdong Second Provincial General Hospital Liu Shuang at the Department of Hematology, Guangdong Second Provincial General Hospital for technical assistance.

Funding

This work was supported by the National Natural Science Foundation of China (no. 81400996) and the Natural Science Foundation of Guangdong Province, China (no. 2016A030313627).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ZZL designed the research, collected and analyzed data, and drafted the manuscript. TXB performed the main part of the experiments. MBL discussed the results and edited the manuscript. XHL contributed to the western blotting and Q-PCR analysis. HLS helped establish the SCI models. HZX, YS, XH and XLW participated in the cell culture and animal behavioral tests. ZH contributed to the conception and design and supervised the study.

All authors critically reviewed and approved the submitted version of the manuscript.

Ethics approval and consent to participate

All experimental procedure was approved by the Guangdong Second Provincial General Hospital Ethics Committee, GuangZhou, China. All protocols were performed to minimize pain or discomfort.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Figure legends

Fig 1. ADSCs mitigated macrophage infiltration and inflammatory cytokine expression following SCI

(A) Representative images of Iba1 and ED-1+ cells in the sham, ADSCs, and control groups at 7 days following SCI. (B) Quantification of Iba1- and ED-1+ cells in the different groups. ADSCs treatment significantly decreased the fluorescence intensity of Iba1- and ED-1+ cells compared to that in the control group (n=4; the data are expressed as the mean \pm SD; * P <0.05 versus the sham group; # P <0.05 versus the Control group). (C) Q-PCR analysis of the expression of IL-6, TNF- α and IL-1 β . The results are expressed as the fold increase compared with the level expressed in the sham group (the values are expressed as the means \pm SEM; * P < 0.05 versus the sham group; # P <0.05 versus the Control group). Scale bar, 50 μ m.

Fig 2. ADSC treatment increased the survival of neurons after SCI. (A) Representative fluorescence microscope images of NeuN+ neurons in the ventral horn at 7 days after SCI. Representative images were taken 2 mm rostral to the lesion epicenter. Scale bar, 100 μ m. (B) The relative number of neurons with strong NeuN+ staining at days 3, 7, 14 and 28 post-SCI (* P <0.05 versus the Control group). (C) Representative images of activated caspase-3/NeuN+ neurons in the ventral horns of control and SCI mice. The activated caspase-3+ neurons showed weak fluorescence staining for NeuN, while the activated caspase-3-neurons showed obvious fluorescence staining. Scale bar, 100 μ m. (D) The proportion of NeuN+ neurons that were also positive for activated caspase-3 (* P <0.01). (E) ADSC treatment promoted the recovery of locomotor capacity of SCI mice, as evaluated by BMS scores at different time points (* P <0.05).

Fig 3. The Notch pathway was activated after SCI. (A) Western blot analysis showing that NICD expression began to increase at 1 day and reached its peak expression at 3 days post-SCI. β -actin was used as a loading control. (B) Quantification of NICD expression (n = 4 in each group; the data are expressed as the mean \pm SEM; ** P < 0.05 versus the sham group) (C) Immunostaining for notch 1 and NICD at day 3 after SCI. The dotted lines indicate the outline of the ventral horn, Scale bar, 100 μ m.

Fig 4. Astrocytes and neurons were implicated in Notch1/Jagged1 signaling pathway activation after SCI.

Double immunofluorescence staining for NICD and Jagged 1 in the ventral horn of the spinal cord of SCI mice at 3 days after SCI showed that NICD was mainly expressed in NeuN+ neurons (A) but not in GFAP+ astrocytes (C). Jagged 1 was mainly expressed in GFAP+ astrocytes (B). Scale bar, 100 μ m. The arrowheads show the processes of GFAP+ astrocytes surrounding NICD+ cells (full arrows). Scale bar, 40 μ m.

Fig 5. ADSCs treatment significantly inhibited the SCI-induced upregulation of Notch1 pathway-related mediated factors at 3 days post-SCI. (A) Representative western blots for Notch1 signaling pathway-associated proteins after ADSC treatment at 3 days postinjury and in the uninjured spinal cord. (B) Representative NICD staining images and the quantification of NICD at 3 days postinjury compared to that in the uninjured spinal cord (*P < 0.05 versus the sham group; #P < 0.05 versus the control group; n = 4, Scale bar, 50 μ m). Quantification of the relative expression levels of NICD (C), Jagged1 (D) and RBP-JK (E) normalized to the level of β -actin (the data are expressed as the mean \pm SEM; *P < 0.05 versus the sham group; #P < 0.05 versus the control group; n = 4).

Fig 6. Jagged1 siRNA inhibited the Jagged1/Notch signaling pathway and alleviated the neuroinflammatory response at 3 days post-SCI. (A) Representative western blot images showing that Jagged1 siRNA significantly reduced the expression of Jagged1, NICD and RBP-JK. Quantification of the relative expression levels of

Jagged1 (B), NICD (C) and RBP-JK (D) normalized to the level of β -actin (the data are expressed as the mean \pm SEM; *P < 0.05 versus the sham group, #P < 0.05 versus the Jagged1 siRNA group; n = 4). (E) Representative images of Iba1+ cells in the control, scramble siRNA, sham and Jagged1 siRNA groups at 3 days after SCI (F) Quantification of Iba1+ cells in the different groups. (the data are expressed as the mean \pm SD; *P<0.05 versus the sham group; #P<0.05 versus the Jagged1 siRNA group; n=4, Scale bar, 100 μ m). (G) Q-PCR analysis of the mRNA expression of IL-6, IL-1 β and TNF- α . The results are expressed as the fold increase compared with the level expressed in the sham group (the values are expressed as the means \pm SEM; *P < 0.05 versus the sham group; #P<0.05 versus the Jagged1 siRNA group).

Fig 7. Jagged1 inhibition induced downregulation of the JAK/STAT3 signaling pathway. (A) Representative western blot images showing the protein levels of JAK/STAT3 pathway-related molecules at 3 days after Jagged1 siRNA injection. Quantification of STAT3 (B), p-STAT3 (C) and JAK (D) (the data are expressed as the mean \pm SEM; *P < 0.05 versus the sham group; #P < 0.05 versus the SCI+ Scramble siRNA group; n=4).

References:

1. Fitzharris M, Cripps RA, Lee BB: **Estimating the global incidence of traumatic spinal cord injury.** *Spinal Cord* 2014, **52**:117-122.
2. Sofroniew MV: **Dissecting spinal cord regeneration.** *Nature* 2018, **557**:343-350.
3. Gensel JC, Zhang B: **Macrophage activation and its role in repair and pathology after spinal cord injury.** *Brain Res* 2015, **1619**:1-11.
4. Qu J, Zhang H: **Roles of Mesenchymal Stem Cells in Spinal Cord Injury.** *Stem Cells Int* 2017, **2017**:5251313.

5. Zhou Z, Chen Y, Zhang H, Min S, Yu B, He B, Jin A: **Comparison of mesenchymal stromal cells from human bone marrow and adipose tissue for the treatment of spinal cord injury.** *Cytotherapy* 2013, **15**:434-448.
6. Cunningham CJ, Redondo-Castro E, Allan SM: **The therapeutic potential of the mesenchymal stem cell secretome in ischaemic stroke.** *J Cereb Blood Flow Metab* 2018, **38**:1276-1292.
7. Cheng Z, Wang L, Qu M, Liang H, Li W, Li Y, Deng L, Zhang Z, Yang GY: **Mesenchymal stem cells attenuate blood-brain barrier leakage after cerebral ischemia in mice.** *J Neuroinflammation* 2018, **15**:135.
8. Fernandez O, Izquierdo G, Fernandez V, Leyva L, Reyes V, Guerrero M, Leon A, Arnaiz C, Navarro G, Paramo MD, et al: **Adipose-derived mesenchymal stem cells (AdMSC) for the treatment of secondary-progressive multiple sclerosis: A triple blinded, placebo controlled, randomized phase I/II safety and feasibility study.** *Plos One* 2018, **13**:e195891.
9. Cheng H, Liu X, Hua R, Dai G, Wang X, Gao J, An Y: **Clinical observation of umbilical cord mesenchymal stem cell transplantation in treatment for sequelae of thoracolumbar spinal cord injury.** *J Transl Med* 2014, **12**:253.
10. Dabrowska S, Andrzejewska A, Lukomska B, Janowski M: **Neuroinflammation as a target for treatment of stroke using mesenchymal stem cells and extracellular vesicles.** *J Neuroinflammation* 2019, **16**:178.
11. Zhou Z, Chen Y, Zhang H, Min S, Yu B, He B, Jin A: **Comparison of mesenchymal stromal cells from human bone marrow and adipose tissue for the treatment of spinal cord injury.** *Cytotherapy* 2013, **15**:434-448.
12. Stump G, Durrer A, Klein AL, Lutolf S, Suter U, Taylor V: **Notch1 and its ligands Delta-like and Jagged are expressed and active in distinct cell populations in the postnatal mouse brain.** *Mech Dev* 2002, **114**:153-159.
13. Kopan R, Ilagan MX: **The canonical Notch signaling pathway: unfolding the activation mechanism.** *Cell* 2009, **137**:216-233.
14. Ben HL, Ceyzeriat K, Carrillo-de SM, Aubry F, Auregan G, Guillermier M, Ruiz M, Petit F, Houitte D, Faivre E, et al: **The JAK/STAT3 pathway is a common inducer of astrocyte reactivity in Alzheimer's and Huntington's diseases.** *J Neurosci* 2015, **35**:2817-2829.
15. Justicia C, Gabriel C, Planas AM: **Activation of the JAK/STAT pathway following transient focal cerebral ischemia: signaling through Jak1 and Stat3 in astrocytes.** *Glia* 2000, **30**:253-270.
16. Wanner IB, Anderson MA, Song B, Levine J, Fernandez A, Gray-Thompson Z, Ao Y, Sofroniew MV: **Glial scar borders are formed by newly proliferated, elongated astrocytes that interact to corral inflammatory and fibrotic cells via STAT3-dependent mechanisms after spinal cord injury.** *J Neurosci* 2013, **33**:12870-12886.
17. Herrmann JE, Imura T, Song B, Qi J, Ao Y, Nguyen TK, Korsak RA, Takeda K, Akira S, Sofroniew MV: **STAT3 is a critical regulator of astrogliosis and scar formation after spinal cord injury.** *J Neurosci* 2008, **28**:7231-7243.
18. LeComte MD, Shimada IS, Sherwin C, Spees JL: **Notch1-STAT3-ETBR signaling axis controls reactive astrocyte proliferation after brain injury.** *Proc Natl Acad Sci U S A* 2015, **112**:8726-8731.
19. Qian D, Li L, Rong Y, Liu W, Wang Q, Zhou Z, Gu C, Huang Y, Zhao X, Chen J, et al: **Blocking Notch signal pathway suppresses the activation of neurotoxic A1 astrocytes after spinal cord injury.** *Cell Cycle* 2019:1-20.
20. Morga E, Mouad-Amazzal L, Felten P, Heurtaux T, Moro M, Michelucci A, Gabel S, Grandbarbe L, Heuschling P: **Jagged1 regulates the activation of astrocytes via modulation of NFkappaB and JAK/STAT/SOCS pathways.** *Glia* 2009, **57**:1741-1753.
21. Liu W, Li R, Yin J, Guo S, Chen Y, Fan H, Li G, Li Z, Li X, Zhang X, et al: **Mesenchymal stem cells alleviate the early brain injury of subarachnoid hemorrhage partly by suppression of Notch1-dependent neuroinflammation: involvement of Botch.** *J Neuroinflammation* 2019, **16**:8.

22. Xing Y, Chen X, Cao Y, Huang J, Xie X, Wei Y: **Expression of Wnt and Notch signaling pathways in inflammatory bowel disease treated with mesenchymal stem cell transplantation: evaluation in a rat model.** *Stem Cell Res Ther* 2015, **6**:101.
23. Liu S, Liu D, Chen C, Hamamura K, Moshaverinia A, Yang R, Liu Y, Jin Y, Shi S: **MSC Transplantation Improves Osteopenia via Epigenetic Regulation of Notch Signaling in Lupus.** *Cell Metab* 2015, **22**:606-618.
24. Bellver-Landete V, Bretheau F, Mailhot B, Vallieres N, Lessard M, Janelle ME, Vernoux N, Tremblay ME, Fuehrmann T, Shoichet MS, Lacroix S: **Microglia are an essential component of the neuroprotective scar that forms after spinal cord injury.** *Nat Commun* 2019, **10**:518.
25. Basso DM, Fisher LC, Anderson AJ, Jakeman LB, McTigue DM, Popovich PG: **Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains.** *J Neurotrauma* 2006, **23**:635-659.
26. O'Shea TM, Burda JE, Sofroniew MV: **Cell biology of spinal cord injury and repair.** *J Clin Invest* 2017, **127**:3259-3270.
27. Scolding NJ, Pasquini M, Reingold SC, Cohen JA: **Cell-based therapeutic strategies for multiple sclerosis.** *Brain* 2017, **140**:2776-2796.
28. Weston NM, Sun D: **The Potential of Stem Cells in Treatment of Traumatic Brain Injury.** *Curr Neurol Neurosci Rep* 2018, **18**:1.
29. Assinck P, Duncan GJ, Hilton BJ, Plemel JR, Tetzlaff W: **Cell transplantation therapy for spinal cord injury.** *Nat Neurosci* 2017, **20**:637-647.
30. Chung TN, Kim JH, Choi BY, Chung SP, Kwon SW, Suh SW: **Adipose-derived mesenchymal stem cells reduce neuronal death after transient global cerebral ischemia through prevention of blood-brain barrier disruption and endothelial damage.** *Stem Cells Transl Med* 2015, **4**:178-185.
31. Yu Z, Wenyan T, Xuewen S, Baixiang D, Qian W, Zhaoyan W, Yinxiang Y, Suqing Q, Zuo L: **Immunological effects of the intraparenchymal administration of allogeneic and autologous adipose-derived mesenchymal stem cells after the acute phase of middle cerebral artery occlusion in rats.** *J Transl Med* 2018, **16**:339.
32. Li YX, Sibon O, Dijkers PF: **Inhibition of NF-kappaB in astrocytes is sufficient to delay neurodegeneration induced by proteotoxicity in neurons.** *J Neuroinflammation* 2018, **15**:261.
33. Liddelow SA, Guttenplan KA, Clarke LE, Bennett FC, Bohlen CJ, Schirmer L, Bennett ML, Munch AE, Chung WS, Peterson TC, et al: **Neurotoxic reactive astrocytes are induced by activated microglia.** *Nature* 2017, **541**:481-487.
34. Hao XZ, Yin LK, Tian JQ, Li CC, Feng XY, Yao ZW, Jiang M, Yang YM: **Inhibition of Notch1 Signaling at the Subacute Stage of Stroke Promotes Endogenous Neurogenesis and Motor Recovery After Stroke.** *Front Cell Neurosci* 2018, **12**:245.
35. Dias TB, Yang YJ, Ogai K, Becker T, Becker CG: **Notch signaling controls generation of motor neurons in the lesioned spinal cord of adult zebrafish.** *J Neurosci* 2012, **32**:3245-3252.
36. Arumugam TV, Chan SL, Jo DG, Yilmaz G, Tang SC, Cheng A, Gleichmann M, Okun E, Dixit VD, Chigurupati S, et al: **Gamma secretase-mediated Notch signaling worsens brain damage and functional outcome in ischemic stroke.** *Nat Med* 2006, **12**:621-623.
37. Liu S, Liu D, Chen C, Hamamura K, Moshaverinia A, Yang R, Liu Y, Jin Y, Shi S: **MSC Transplantation Improves Osteopenia via Epigenetic Regulation of Notch Signaling in Lupus.** *Cell Metab* 2015, **22**:606-618.
38. Sun F, Mao X, Xie L, Ding M, Shao B, Jin K: **Notch1 signaling modulates neuronal progenitor activity in the subventricular zone in response to aging and focal ischemia.** *Aging Cell* 2013, **12**:978-987.
39. Nonneman A, Criem N, Lewandowski SA, Nuyts R, Thal DR, Pfriederger FW, Ravits J, Van Damme P, Zwijsen A, Van Den Bosch L, Robberecht W: **Astrocyte-derived Jagged-1 mitigates deleterious Notch signaling in amyotrophic**

lateral sclerosis. *Neurobiol Dis* 2018, **119**:26-40.

40. Stump G, Durrer A, Klein AL, Lutolf S, Suter U, Taylor V: **Notch1 and its ligands Delta-like and Jagged are expressed and active in distinct cell populations in the postnatal mouse brain.** *Mech Dev* 2002, **114**:153-159.