LncRNA Airsci increases the inflammatory response after spinal cord injury in rats through the nuclear factor kappa B signaling pathway

Abstract
Spinal cord injury (SCI) is a serious traumatic event to the central nervous system. Studies show that long non-coding RNAs (lncRNAs) play an important role in regulating the inflammatory response in the acute stage of SCI. Here, we investigated a new lncRNA related to spinal cord injury and acute inflammation. We analyzed the expression profile of lncRNAs after SCI, and explored the role of lncRNA Airsci (acute inflammatory response in SCI) on recovery following acute SCI. The rats were divided into the control group, SCI group, and SCI + lncRNA Airsci-siRNA group. The expression of inflammatory factors, including nuclear factor kappa B [NF-κB (p65)], NF-κB inhibitor IκBα and phosphorylated IκBα (p-IκBα), and the p-IκBα/IκBα ratio were examined 1–28 days after SCI in rats by western blot assay. The differential lncRNA expression profile after SCI was assessed by RNA sequencing. The differentially expressed lncRNAs were analyzed by bioinformatics technology. The differentially expressed lncRNA Airsci, which is involved in NF-κB signaling and associated with the acute inflammatory response, was verified by quantitative real-time PCR. Interleukin (IL-1β), IL-6 and tumor necrosis factor (TNF-α) at 3 days after SCI were measured by western blot assay and quantitative real-time PCR. The histopathology of the spinal cord was evaluated by hematoxylin-eosin and Nissl staining. Motor function was assessed with the Basso, Beattie and Bresnahan Locomotor Rating Scale. Numerous differentially expressed lncRNAs were detected after SCI, including 151 that were upregulated and 186 that were downregulated in the SCI 3 d group compared with the control group. LncRNA Airsci was the most significantly expressed among the five lncRNAs involved in the NF-κB signaling pathway. LncRNA Airsci-siRNA reduced the inflammatory response by inhibiting the NF-κB signaling pathway, alleviated spinal cord tissue injury, and promoted the recovery of motor function in SCI rats. These findings show that numerous lncRNAs are differentially expressed following SCI, and that inhibiting lncRNA Airsci reduces the inflammatory response through the NF-κB signaling pathway, thereby promoting functional recovery. All experimental procedures and protocols were approved by the approved by the Animal Ethics Committee of Jining Medical University (approval No. JNMC-2020-DW-RM-003) on January 18, 2020.

Key Words: apoptosis; functional recovery; inflammatory response; long non-coding RNA; neuroprotection; NF-κB signaling pathway; RNA sequencing; spinal cord injury

Introduction
Spinal cord injury (SCI) can lead to severe dysfunction of the limbs below the damaged segment, which not only affects the physical and mental health and quality of life of patients, but also imparts a huge economic burden on families and society (Kumar et al., 2018; Jung et al., 2019). The pathogenesis of SCI is complex, involving two stages of primary and secondary injury (Cao and Krause, 2020). After the occurrence of primary injuries such as fracture and compression, a series of secondary injuries such as inflammation, hypoxia, neuronal...
necrosis and apoptosis, and local microenvironmental changes further aggravate the SCI, resulting in severe loss of sensory and motor functions (Dai et al., 2018; Li et al., 2019b; Lv et al., 2019). At present, clinical therapeutics for SCI are limited and the pathogenesis of SCI still needs to be further studied.

It has been shown that the inflammatory response plays a key role in the pathogenesis of SCI (do Espírito Santo et al., 2019; He et al., 2019; Wang et al., 2019; Oh et al., 2020). Dysregulation of the inflammatory response after SCI causes a cascade effect, inducing apoptosis, edema, oxidative stress, and other reactions that aggravate tissue damage (Gao et al., 2019; Huang et al., 2019). Early anti-inflammatory treatment can improve the clinical prognosis of patients with SCI (Gensel and Zhang, 2015). It has also been found that stem cell transplantation in the acute inflammatory phase can provide better functional recovery (Nishimura et al., 2013; Cheng et al., 2017). Therefore, an in-depth study of the molecular mechanisms of SCI, including the dysregulation of the inflammatory response, may provide new molecular targets for the diagnosis and treatment of SCI.

Long non-coding RNA (lncRNA) is a type of RNA with a transcript length of over 200 nt and no protein-coding function (Rui et al., 2018). LncRNAs are widely expressed in organisms and have important regulatory functions (McDonel and Guttman, 2019; Qian et al., 2019). The main functions of IncRNAs include participating with competitive endogenous RNAs, interacting with proteins, regulating mRNA shearing, chromatin and histone remodeling, and transcriptional regulation (Shi et al., 2018). Preliminary studies show that IncRNAs play a critical role in the regulation of the molecular mechanisms involved in the acute phase of inflammatory dysregulation in SCI (Shao et al., 2020). LncRNA LOC685699-OT1 is a new transcript of gene LOC685699 (ENSRNOG00000043358), located on chromosome X:23419209–23467865. No studies on lncRNA LOC685699-OT1 in SCI have been found to date. Therefore, we named this LncRNA Airsci (acute inflammatory response in SCI) according to the rules of the Hugo Gene Naming Committee (HGNC) naming guidelines. Here, we found that IncRNA Airsci had the most significantly differential expression after SCI in rats, and its expression was significantly up-regulated during the acute inflammatory phase. Hence, exploring the molecular functions of IncRNA Airsci in SCI may provide new therapeutic targets and intervention strategies for treatment and rehabilitation following SCI.

### Materials and Methods

#### Animals

A total of 105 healthy and specific pathogen-free adult (6–7-week-old) male Sprague-Dawley (SD) rats, weighing 250–280 g, were purchased from Jinan Penghui Experimental Animal Breeding Co., Ltd (Jinan, Shandong Province, China). The rats were kept at 25 ± 0.5°C in the SPF Experimental Animal Center of Jining Medical University. The light/dark cycle was altered for 12 hours. To examine the regulatory function of IncRNA Airsci in SCI rats, we divided the rats into the control group, SCI group, and SCI + IncRNA Airsci-siRNA group. Control group: only laminectomy, no SCI; SCI group: no laminectomy, SCI; SCI + IncRNA Airsci-siRNA group: The plKO.1-sh Airsci small interfering RNA gene silencing plasmid was constructed. After transformation and identification, the plasmid was amplified using the plasmid extraction kit (Beyotime, Shanghai, China). The plasmid was packaged with lentivirus and transfected in 293T cells according to the plasmid was amplified using the plasmid extraction kit (Beyotime, Shanghai, China). The plasmid was packaged with lentivirus and transfected in 293T cells according to the plasmid was amplified using the plasmid extraction kit (Beyotime, Shanghai, China). The plasmid was packaged with lentivirus and transfected in 293T cells according to the plasmid was amplified using the plasmid extraction kit (Beyotime, Shanghai, China). The plasmid was packaged with lentivirus and transfected in 293T cells according to the plasmid was amplified using the plasmid extraction kit (Beyotime, Shanghai, China). The plasmid was packaged with lentivirus and transfected in 293T cells according to the plasmid was amplified using the plasmid extraction kit (Beyotime, Shanghai, China). The plasmid was packaged with lentivirus and transfected in 293T cells according to the plasmid was amplified using the plasmid extraction kit (Beyotime, Shanghai, China). The plasmid was packaged with lentivirus and transfected in 293T cells according to the plasmid was amplified using the plasmid extraction kit (Beyotime, Shanghai, China). The plasmid was packaged with lentivirus and transfected in 293T cells according to the
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classified after removing ribosomal RNA. The library was
initially quantified by Qubit 2.0 (Shanghai, China) and
diluted to 1.5 ng/μL. Then, the insert size (length of the sequence
between the pair adapters) was analyzed on an Agilent 2100
(Shanghai, China). Once the insert size was satisfactory, the
effective concentration of the library (greater than 3 nM) was
accurately quantified by qRT-PCR to ensure library quality.
Then, Illumina PE150 sequencing was conducted after pooling
of the library according to the coverage required (sequencing
was conducted by Shanghai Jikai Gene Chemical Technology
Co., Ltd., Shanghai, China).

Screening of lncRNAs by bioinformatics technology
We used RNA-sequencing to detect changes in lncRNA
expression in the spinal cord tissues of rats in the control
group and the SCI 3d group, and obtained the expression
profile heat map of lncRNAs. Then, bioinformatics technology
was used to screen the differential lncRNAs in the SCI 3d
group that were related to the inflammatory response. Kyoto
Encyclopedia of Genes and Genomes (KEGG) (https://
www.kegg.jp/) is a database for the systematic analysis of
relationships, gene function and gene information between
genes and their encoded products. To identify lncRNAs
associated with acute inflammation, we first screened lncRNAs
according to the set fold change (over 10 times) and the
significance level of expression difference (< 0.05) between
the SCI 3 days and control groups. Next, target gene prediction
and KEGG function enrichment analysis were performed.
Finally, the lncRNA Airsci, which is involved in the NF-κB
signaling pathway and associated with the acute inflammatory
response, was selected for further analysis.

Quantitative real-time PCR
Quantitative real-time PCR (qRT-PCR) was used to measure the
expression levels of related genes in each group 3 days after SCI.
Total RNA in tissues (40–50 mg) was extracted by the Trizol
method (Invitrogen). The purity and quantity of RNA were
determined with an ultraviolet spectrophotometer (SYNERGY
H1, BioTek, USA) and then diluted with diethylpyrocarbonate
(DEPC) water (Beyotime, Shanghai, China). cDNA was then
synthesized according to the instructions in the TaKaRa RNA
PCR Kit (TaKaRa, Otsu, Japan). qRT-PCR parameters were as follows: 95°C for 5 minutes, followed by 40 cycles
(amplification) of 95°C for 15 seconds (denaturation), 60°C for
40 seconds (annealing), and 72°C for 20 seconds (extension).
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was
used as a reference gene, and the relative quantification
method (2^-ΔΔCt) was used. The sequences of all primers used for the qRT-PCR
experiments are presented in Table 1.

Hematoxylin-eosin and Nissl staining
Hematoxylin-eosin (HE) and Nissl staining were used to
evaluate the histopathology of the spinal cord in each group
at 3 days after SCI. The collected tissues were paraffin-
embedded, sectioned into 10 μm sections, and then HE and
Nissl staining were performed. HE staining: The sections were
stained with hematoxylin for 1 minute, and washed with water.
Then, they were immersed in 1% hydrochloric acid/ethanol
for 5 seconds and rinsed with water. Sections were
then treated with ammonia water for 5 minutes and rinsed
with water. Eosin staining was conducted for 1 min and rinsed
with water. Sections were then dehydrated sequentially
with 80%, 95% and anhydrous ethanol for 10 seconds each.
Nissl: The sections were immersed overnight in an equal
volume of anhydrous ethanol and chloroform solution. On
the next day, they were immersed in anhydrous ethanol, 95%
ethanol, and distilled water. The sections were then placed
in 0.1% methylenephlorin solution at 40°C for 10 minutes and
washed quickly with distilled water. We observed the tissue
morphology of HE staining sections with an optical microscope
(Olympus, Jinan, China) and measured the proportional
spinal cord tissue lesion size with ImageJ software (NIH,
Bethesda, USA). We observed the neurons in the anterior
horn of the spinal cord and performed count analysis with the
optical microscope (five animals were included; counting was
performed at 400x magnification; results were expressed as
averages).

Basso, Beattie and Bresnahan Locomotor Rating Scale
assessment
The motor function of rat hind limbs was measured by the
Basso, Beattie and Bresnahan Locomotor Rating Scale (BBB)
score (All et al., 2020) at different time points (0, 1, 3, 7,
14, 21, 28 days) after SCI. BBB scores ranged from 0 to 21
points. The minimum number of points (0) indicates complete
paralysis and the maximum number of points (21) represents
normal function. Three uninformed researchers assessed the
motor function of rats. As the score increased, the motor
function improved.

Statistical analysis
Data were analyzed using GraphPad Prism version 5.0 for
Windows (GraphPad Software, La Jolla, CA, USA) and expressed
as the mean ± SD. Western blot, qPCR, HE and Nissl staining
differences among multiple groups were analyzed with one-
way analysis of variance and the least significant difference
test. BBB scores were analyzed using the Mann-Whitney U-test.
P < 0.05 was considered statistically significant.

Results
The NF-κB signaling pathway is activated after SCI in rats
Western blot results showed that NF-κB (p65) and p-IκBα
protein expression increased initially and then decreased
between 1 and 28 days after SCI (Figure 1). The p-IκBα/IκBα
ratio initially increased and reached a peak at 3 days after SCI,
and thereafter decreased (Figure 1).

lncRNAs are differentially expressed after SCI in rats
RNA-sequencing results showed that lncRNAs were
differentially expressed at 3 days after SCI (Figure 2A).
Compared with the control group, 151 lncRNAs were
upregulated and 186 lncRNAs were downregulated in the SCI
3d group (Figure 2B).

Screening of lncRNA Airsci and examining its effect on the
NF-κB signaling pathway
KEGG functional enrichment analysis of lncRNA expression
profiles at 3 days after SCI revealed a variety of signaling
pathways, including the NF-κB signaling pathway (Figure 3A).
Bioinformatics was used to screen for lncRNAs involved in
the NF-κB signaling pathway and associated with the acute
inflammatory response. The screen identified the following
five lncRNAs: LOC685699-OT1 (Airsci), AABR07015057.1-OT1,
LIN5298, AABR07003398.1-OT18 and AABR0705878.1-
OT1. All five lncRNAs were verified by qRT-PCR, and the
differences were found to be statistically significant (P < 0.05),
consistent with the results of RNA-sequencing. Among these,
the expression of lncRNA Airsci increased significantly after
SCI (Figure 3B). Accordingly, we divided the rats into the
control group, SCI group and SCI + lncRNA Airsci-siRNA group,
and western blot was used to detect the expression levels of
NF-κB (p65), phospho-IκBα and IκBα (Figure 3C). Inhibition
of lncRNA Airsci significantly reduced the protein expression
levels of NF-κB (p65) and p-IκBα, and reduced the p-IκBα/IκBα
ratio (Figure 3D–G).

Inhibiting lncRNA Airsci reduces the inflammatory response
after SCI in rats
Western blot and qRT-PCR results showed that the expression
levels of IL-1β, IL-6 and TNF-α were dramatically increased in
Inhibiting IncRNA Airsci ameliorates histopathological changes in the spinal cord and promotes the recovery of motor function in rats

HE staining was performed to assess the histopathology of the spinal cord tissues 3 days after SCI (Figure 5A). The proportional lesion size was larger in the SCI group compared with the control group. In comparison, the proportional lesion size was smaller in the Airsci-siRNA group than in the SCI group (Figure 5B). Nissl staining was used for counting spinal cord motor neurons and for detecting neuronal injury (Figure 5A). The number of Nissl-positive cells in the anterior horn of the spinal cord was less in the SCI group compared with the control group. Airsci-siRNA dramatically increased the number of Nissl-positive cells compared with the SCI group (Figure 5C). The BBB score was used to assess motor function after SCI (Figure 5D). The BBB score in the SCI group decreased significantly compared with the control group. Notably, the score was markedly higher in the Airsci-siRNA group than in the SCI group at 7 days after SCI.

Figure 1 | NF-κB signaling pathway expression before and after SCI. (A) Western blot assay was performed to analyze changes in expression of the inflammatory proteins NF-κB (p65), p-IκBα and IκBα, which are associated with the NF-κB signaling pathway, at 1, 2, 3, 5, 7, 14, 21 and 28 days after SCI. (B–E) The optical density analysis of NF-κB (p65), p-IκBα and IκBα protein and the p-IκBα/IκBα ratio at 1, 2, 3, 5, 7, 14, 21 and 28 days after SCI. Data are expressed as the mean ± SD (n = 5, one-way analysis of variance and the least significant difference test). *
P < 0.05, **P < 0.01, ***P < 0.001. IκBα: NF-κB inhibitor; NF-κB: nuclear factor kappa-B; p-IκBα: phosphorylated IκBα; SCI: spinal cord injury.
The inflammatory response plays an important role in the pathophysiology of SCI and affects recovery and functional outcome (Lemmens et al., 2019; Şaker et al., 2019). Studies have shown that lncRNAs play an important role in regulating the inflammatory response in the acute stage of SCI (Zhou et al., 2018; Jia et al., 2019). Therefore, further study of the molecular mechanisms underpinning lncRNA function may provide new ideas and methods for the treatment of SCI.

In this study, our major findings are as follows: (1) The NF-κB signaling pathway is significantly activated after SCI, and the inflammatory response is significantly increased, with the most significant increase at 3 days after SCI; (2) The expression of lncRNA after SCI is significantly different and plays a pivotal role in the pathological mechanism of SCI; (3) LncRNA Airsci is involved in the regulation of the NF-κB inflammatory signaling pathway; (4) Inhibiting lncRNA Airsci reduces the inflammatory response, lessens the area of spinal cord tissue damage, reduces the death of neurons, and promotes the recovery of motor function.

Current studies demonstrate that lncRNAs are significantly differentially expressed after SCI, and play an important regulatory role in the process of tissue repair (Zhang et al., 2018; Ren et al., 2019). For example, lncRNA MALAT1 inhibits autophagy and apoptosis of neurons and promotes SCI repair by regulating the Wnt/β-catenin and PI3K/Akt signaling pathways (Liu et al., 2019). In vitro studies of PC12 cells show that lncRNA Mirt2 inhibits the p38MAPK signaling pathway.
by downregulating miR-429 and reducing apoptosis (Li et al., 2019a). Unfortunately, these studies on lncRNA in SCI are mainly focused on neuronal proliferation, apoptosis, and autophagy, and few studies have conducted studies on the regulatory function of lncRNAs in the acute phase of SCI (Ren et al., 2019).

Preliminary research has shown that IncRNA MALAT1 promotes the inflammatory response in the SCI acute phase by regulating miRNA-199b, demonstrating that IncRNA plays an important role in the regulation of the molecular mechanisms of the acute inflammatory response (Zhou et al., 2018). However, in-depth studies of the expression profile of lncRNAs in the SCI acute phase are lacking. Further study, including additional screening of lncRNAs, may clarify their important regulatory functions in the SCI acute inflammatory response, and may provide new ideas and methods for SCI treatment.

A modified Allen’s apparatus was used to produce the rat SCI model, and we assessed changes in NF-kB(p65), p-κBα, κBα and the p-κBα/κBα ratio at 1, 2, 3, 5, 7, 14, 21 and 28 days after SCI. We found that the NF-kB signaling pathway reached peak activation on day 3 after SCI. We used RNA-seq to detect changes in IncRNA expression in spinal cord tissues after SCI, and found that different lncRNAs were expressed. With the control group, 151 lncRNAs were upregulated and 185 IncRNAs were downregulated in the SCI-3 group. To identify lncRNAs involved in the NF-kB signaling pathway and associated with the acute inflammatory response, we screened the IncRNAs through bioinformatics analysis and then verified them by qRT-PCR. Among the five lncRNAs of interest, IncRNA Airsci was significantly upregulated during the SCI acute inflammatory phase. We hypothesized that exploring the role and mechanism of IncRNA Airsci in SCI may provide new therapeutic targets and intervention strategies for the treatment and rehabilitation of SCI. We found that inhibiting IncRNA Airsci expression could reduce the inflammatory factors IL-1β, IL-6 and TNF-α, and ameliorated spinal cord tissue damage and improved motor function. Therefore, we speculated that inhibiting the expression of IncRNA Airsci could reduce the inflammatory response through NF-kB signaling and promote functional recovery in SCI rats. However, the underlying progression of SCI is closely related to the regulation of the inflammatory response, as well as the differential expression of lncRNAs (Ding et al., 2020). We screened IncRNAs by bioinformatics and verified candidate genes by qRT-PCR. lncRNA Airsci was significantly upregulated during the SCI acute inflammatory phase. Notably, inhibition of IncRNA Airsci reduced the inflammatory response through the NF-kB signaling pathway and promoted functional recovery. Therefore, we speculate that IncRNA Airsci mediates the inflammatory response after SCI in rats through the NF-kB signaling pathway.

In summary, the pathological progression of SCI is closely related to the regulation of the inflammatory response, as well as the differential expression of lncRNAs. Further studies will perform expression analysis on the brain and spinal cord by tissue hybridization to provide insight into the expression profile of Airsci in the CNS in the future.

Acknowledgments: We thank Dong-Mei Shi from Department of the Key Laboratory of Fungi in the Jining First People’s Hospital, China for providing valuable technical assistance in this work.

Author contributions: YZ implemented the experiment and wrote the manuscript, XL and ZL2 collected and analyzed the data, KG and CLL designed and reviewed this manuscript. All authors approved the final version of the manuscript.

Conflicts of interest: The authors declare that they have no conflicts of interest.

Financial support: This work was supported by the National Natural Science Foundation of China (No. 81801908; to KG) and the Natural Science Foundation of Shandong Province of China (No. ZR2018HPN024; to KG).

Institutional review board statement: All experimental procedures and protocols were approved by the approved by the Animal Ethics Committee of Jining Medical University (approval No. JNMU-2020-DW-003) on January 18, 2020. All experimental procedures described here were in accordance with the National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

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Ali AA, Al Nasbahi H, A limited experimental procedures described here were in accordance with the National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

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