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Umbilical mesenchymal stem cell-derived exosomes promote spinal cord functional recovery through the miR-146b/TLR4 -mediated NF-κB p65 signaling pathway in rats

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ABSTRACT

Spinal cord injury (SCI) is an incurable central nervous system impairment that lack of efficient treatment. Exosomes derived from mesenchymal stem cells (MSCs) are widely applied in disease treatment. This work aimed to determine the promising therapeutic effects of MSC-derived exosomal miRNA146b on SCI. A rat spinal cord injury (SCI) model and lipopolysaccharide (LPS)-induced PC12 cell model were established. Exosomes were extracted from human umbilical cord mesenchymal stem cells (hUCMSCs). The identification of exosomes was performed by using transmission electronic microscope (TEM) and nanoparticle tracking analysis (NTA). Hematoxylin and eosin (HE) staining and TUNEL assay were performed to assess tissue damage and apoptosis, respectively. ELISA was performed to detect levels of inflammatory cytokines. Cell viability was checked by cell counting kit 8 (CCK-8). Gene expression and protein levels were detected by qPCR and western blotting assay. The interaction between miR-146 b and Toll-like receptor 4 (TLR4) was assessed by luciferase reporter gene assay. The hUCMSC-derived exosomes could notably alleviate the spinal cord injury and cell apoptosis. The exosomal miR-146 b treatment suppressed the release of IL-1 β, IL-6, and TNFα. The miR-146 b suppressed the expression of TLR4, directly interact with the 3′-untranslated region (3′UTR) of TLR4, and inactivated the nuclear factor κB (NF-κB) signaling. The hUCMSCs-derived exosomal miR-146 b protects neurons from spinal cord injury through targeting the TLR4 and inactivating the NF-κB signaling. Our findings supported the application of hUCMSCs-derived exosomal miR-146 b for the protection of SCI.

1. Introduction

Spinal cord injury (SCI) is an incurable central nervous system impairment and has become a serious burden for individuals both physiologically and psychologically [1,2]. The prevalent treatments for SCI include surgery, traditional drug treatment, tissue engineering, gene therapy, as well as cell therapy [3–5]. Nevertheless, these current treatments only partially repair SCI by improving symptoms and reducing complications. It has been well-established that SCI commonly induce secondary injury such as apoptosis, autophagy, inflammation, and glial scar, which create a chemical and physical barrier to impede neuron regeneration [6,7]. Since the SCI-induced neuron damages are almost irreversible due to limited therapeutic manners, the prevention and management of SCI has become an imperative issue in medical research area.

Mesenchymal stem cells (MSCs) are widely studied form of stem cells that present the self-renewal ability and differentiation ability, hence are recognized as promising therapeutic method for multiple diseases, especially the tissue regeneration [8]. An increasing number of studies have revealed the potential neuroprotective effects of MSCs, including promoting angiogenesis, neurogenesis, matrix remodeling [9]. MSCs mainly include bone marrow mesenchymal stem cells (BMSCs), human umbilical cord mesenchymal stem cells (hUCMSCs), and adipose derived mesenchymal stem cells and have been verified as potential therapy for SCI [10,11]. Exosomes are extracellular vehicles that secreted by cells and contain a variety of signaling factors such as lipid, proteins, and nuclear acid [12]. The exosomes secreted by MSCs have shown critical role in modulating various biological processes [13,14]. Among the cargoes delivered by exosomes, microRNAs (miRNAs) are widely studied [15].

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MiRNAs are small noncoding RNAs that consist of about 22 nuclear acid and cannot encode proteins [16]. MiRNAs usually function as sponge to directly interact with targeted mRNAs and impede gene translation or induce mRNA degradation [17,18]. Accumulating evidence have demonstrated that miRNAs participate in the regulation of cell proliferation, migration, apoptosis, and stress response, and so on [19,20]. For example, miR-216a-5p is enriched in exosomes from hypoxia-induced MSCs and potentially participates in microglial polarization during SCI [21]. Cai and colleagues reported that miR-155 derived from M1 macrophages impairs SCI-induced mitochondrial dysfunction in vascular endothelial cells [22].

Toll-like receptors (TLRs) is a pivotal transmembranous pattern recognition receptor family in innate immune responses and activates the nuclear factor κB (NF-κB) to promote inflammatory response [23]. The activation of TLRs induces the nuclear localization of NF-κB and the transcription of downstream genes such as inflammatory cytokines, which are involved in the regulation of inflammation signaling and inflammatory microenvironment upon SCI injury [24,25].

In this work, we explored the protective effects of exosomal miR-146 b released from hUCMSCs in SCI. We established in vivo rat SCI model and LPS-stimulated in vitro cell model to demonstrated that hUCMSCs-derived exosomal miR-146 b alleviated SCI-induced spinal cord injury and production of inflammation cytokines via targeting the TLR4/NF-κB regulatory axis. Our findings may provide novel evidence for application of MSC-derived exosomes in SCI repairment.

2. Materials and methods

2.1. Cell lines and identification

Human umbilical cord MSCs (hUCMSCs) and PC12 cells were obtained from Wuhan Procell (China). The hUCMSCs were maintained in HUMSC medium that contains 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin/streptomycin. PC12 cells were cultured in IMDM complete medium. All cells were kept in 37 °C incubator that filled with 5% CO_2.

2.2. Animal model

Male (Sprague-Dawley) SD rats that aged 8-weeks old were bought from Beijing Vital River Laboratory (China). The spinal cord ischemia/reperfusion (SCI) model was prepared referring to previous article [26]. In short, the rats were anesthetized using chloral hydrate (10%, 3 ml/kg) and a 5-cm incision was made to expose the renal artery, which was ligated by 2 10-g bulldog clamps for 1 h to occlude blood. The clamps were then removed, and abdominal cavity was closed. The rats in sham group received same operation without blood occlusion. The SCI model was regarded as successful upon the observation of defected motor function in hindlimb. For treatment, a total of 20 μg exoosmes in 10 μL PBS were intrathecally injected 3 days before the surgery. The motor function of hindlimb was analyzed by the Basso, Beattie, and Bresnahan (BBB) grading scale at 24 h post-operation. The spinal cord (L2 to L5) tissues were obtained at 14 days after operation and used for following analysis. All animal experiments in this work were performed according to the guideline of Ethics Committee of Everunion Biotechnology Co. LTD, Tianjin (Approval No. 202103012). All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

2.3. Histopathological evaluation

The spinal cord tissues were sliced into 5 μm-thickness paraffin-embedded samples. The tissues damage was evaluated by hematoxylin and eosin staining kit (ab245880, Abcam, USA). Cell apoptosis of was analyzed by TUNEL assay kit (T2130, Solarbio, China) following the manufacturer's protocol. Images were captured under microscope (SP8, Leica, Germany).

2.4. Flow cytometry

The identification of hUCMSCs was conducted using flow cytometry. The cells were incubated with Alexia fluor specific antibodies against CD34 (ab23830, Abcam, USA), CD44 (ab81424, Abcam, USA), CD45 (ab28106, Abcam, USA), and CD73 (ab157335, Abcam, USA), respectively. After washing with PBS, the samples were examined by a C6 flow cytometer (BD Bioscience, USA).

2.5. Cell treatment and transfection

To mimic inflammatory response of SCI, the PC12 cells were stimulated with lipopolysaccharides (LPS; 5 μg/ml; L2630, Sigma, USA) for 24 h. For inhibition of NF-κB, the PDTC (20 nM; P8765, Merck, USA) was added to PC12 cells for 24 h.

The miR146b mimics, miR-146 b inhibitors, TLR4 overexpression vectors, negative control mimics and empty vectors were bought from GenePharma (Shanghai, China) and transfected by Lipofectamine 2000 reagent (11 668 019, Invitrogen, USA) as per manufacturer's protocol.

2.6. Cell viability

Cell viability was measured by Cell Counting Kit-8 (CCK-8, CA1210, Solarbio, China) as per the protocol. Cells were placed into 96-well plate with 3000 cells in each well and cultured for 24 h. After that, CCK-8 reagent was added into each well and reacted for 2 h. The optical density at 450 nm was detected using a microplate reader (A51119500C, Thermo, USA).

2.7. Isolation and identification of exosomes

The hUCMSCs were cultured in exosome-free medium for 48 h. The exosomes were isolated from the culture medium of hUCMSCs by using standard ultracentrifugation method. In brief, the medium was centrifuged for 20 min at 2000 g to remove debris, then centrifuged at 10 000 g and 4 °C for 30 min. The supernatants were then transferred to a clean ultracentrifuge tube and ultracentrifuged at 100, 000 g and 4 °C for 70 min. After discarding the supernatants, the deposited exosomes were resuspended in PBS, and then ultracentrifuged at 100 000 g and 4 °C for another 70 min. The purified exosomes were resuspended with PBS and were stored at −80 °C for following experiments. The protein content of the collected exosomes was determined using a bicinchoninic acid (BCA) protein assay kit (P0012, Beyotime, China) according to the manufacturer's protocol. The exosome morphology was captured under a Transmission electron microscopy (TEM) (HT7800, Hitachi, Japan). Nanoparticle tracking analysis (NTA) (NanoSight NS300, Malvin, Germany) was conducted to evaluate particle size.

2.8. Exosome internalization

To determine the uptake of exosomes by PC12 cells, the exosomes were probed by PKH-26 (PKH26GL, Sigma, USA) according to the manufacturer's protocol and incubated with PC12 cells for 24 h. Then, the nuclei were labeled with DAPI (Sigma, USA) for 20 min at room temperature. The fluorescence was observed under a confocal microscope (SP8, Leica, Germany).

2.9. Western blot assay

Tissues and cells were lysed by RIPA lysis buffer (P0013b, Beyotime, China). Proteins were separated using SDS-PAGE and blotted onto PVDF membranes (Millipore, USA). The blots were then blocked with 5% non-fat milk and incubated with specific primary antibodies against CD9

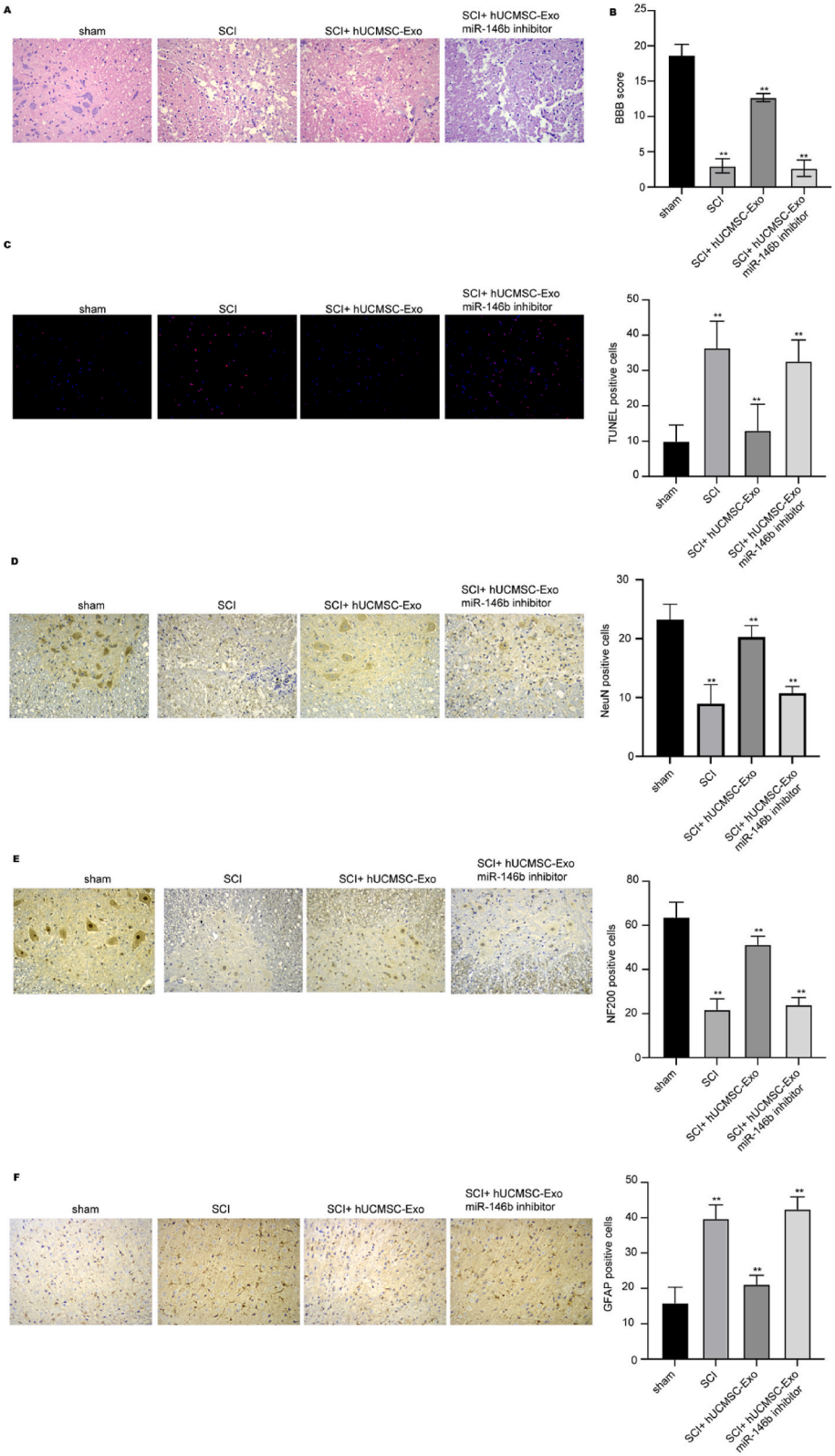

Fig. 1. HUCMSCs -derived exosomal miR-146 b alleviates SCI in rat. SCI rat model was established, and rats were treated with hUCMSCs-derived exosomes. The spinal cord tissues were collected 14 days after operation for histological analysis. (A) The spinal cord tissue damage was measured by HE staining. (B) The hind-limb motor function was checked by BBB score 24 h after operation. (C) Cell apoptosis was measured by TUNEL assay. (D–F) The expression of NeuN, NF200, and GFAP were measured by immunohistochemistry staining. **p < 0.01. Scale bar, 50 μm.

(ab307085, Abcam, USA), CD63 (ab134045, Abcam, USA), CD81 (ab79559, Abcam, USA), TSG101 (ab125011, Abcam, USA), Calnexin (ab133615, Abcam, USA), Bcl-2 (ab32124, Abcam, USA), Bax (ab32503, Abcam, USA), cleaved Caspase-3 (ab2303, Abcam, USA), Caspase-3 (ab32351, Abcam, USA), GFAP (ab7260, Abcam, USA), NF200 (ab134306, Abcam, USA), TLR4 (ab13556, Abcam, USA), p65 (ab32536, Abcam, USA), IκB (9242, CST, USA), pIκB (9246, CST, USA), H3 (ab1791, Abcam, USA), β-actin (ab8226, Abcam, USA) overnight at 4 °C. Next day, the blots were probed with HRP-conjugated anti-rabbit (ab205718, Abcam, USA) or anti-mouse (ab205719, Abcam, USA) secondary antibodies at room temperature for 1 h. After reaction with ECL reagent (Millipore, USA), the protein bands were visualized by a gel

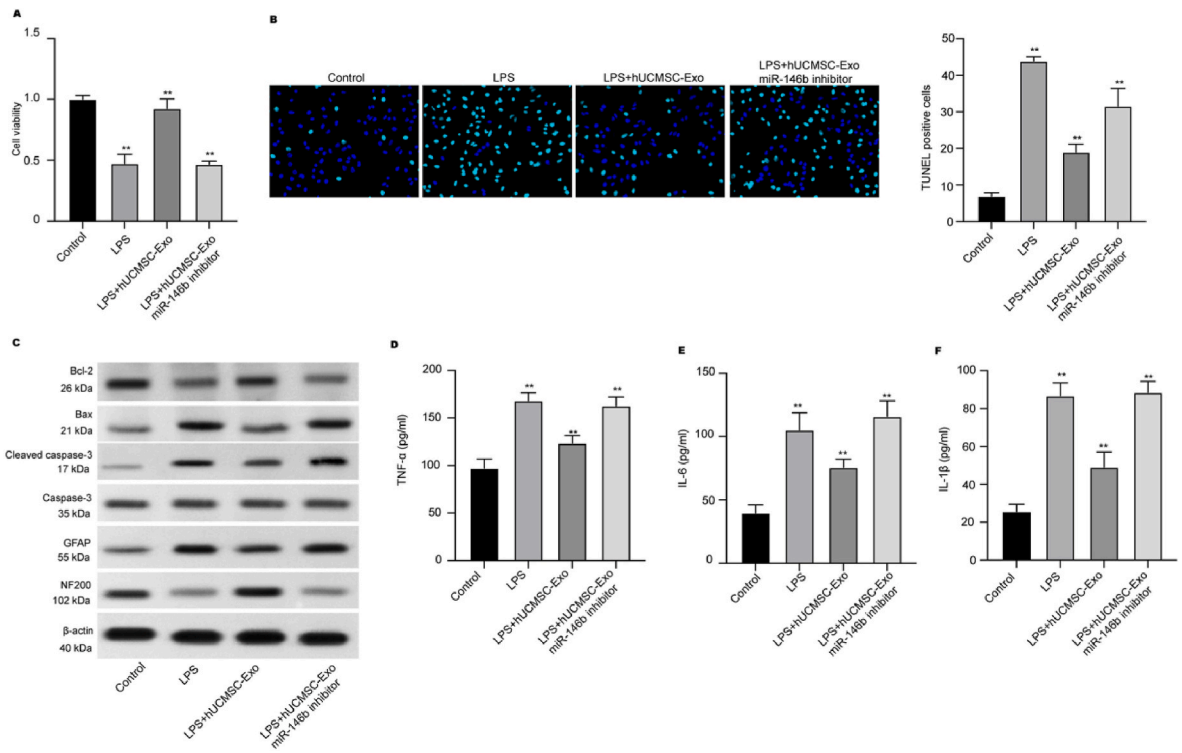

Fig. 2. HUCMSCs -derived exosomal miR-146 b attenuates PC12 nerve cell injury. PC12 cells were stimulated with LPS for 24 h and treated with hUCMSCs -derived exosomes for another 24 h. (A) Cell viability was measured by CCK-8. (B) Cell apoptosis was checked by TUNEL assay. (C) The protein levels of Bcl-2, Bax, cleaved caspase-3, caspase-3, GFAP, and NF200 were detected by western blotting. (D–F) The release of TNF-α, IL-6, and IL-1β were measured by ELISA assay. $^{**}p < 0.01$. Scale bar, 50 μm.

image system.

2.10. Enzyme-linked immune sorbent assay (ELISA)

The levels of TNF-α (KHC3011, Thermo, USA), IL-6 (EH2IL6, Thermo, USA), and IL-1 β (E-EL-H0088c, Elabscience, USA) in tissue lysates and cell culture medium were detected by using commercial ELISA assay kit following manufacturer's protocols.

2.11. Quantitative real-time PCR (qPCR)

The spinal cord tissues and cells were lysed with Trizol reagent (Invitrogen, USA) to isolate total RNA. Then cDNA was synthesized using First-strand synthesis kit (6210 A, Takara, Japan) and gene expression was measured by qPCR using TB Green Premix *Taq*II (RR820Q, Takara, Japan). The RNA levels were normalized to GAPDH or U6.

2.12. Luciferase reporter gene assay

The wild-type (WT) or mutant (MUT) sequences of TLR4 were synthesized and inserted into the pmirGLO vectors. Then, the vectors were co-transfected with miR146b into PC12 cells for 48 h. After that, the cells were lysed, and luciferase activity was examined by a Dual luciferase reporter gene assay system (E1910, Promega, USA).

2.13. Statistics

Data were presented as mean ± SD of at least three independent replicates and analyzed using SPSS 20.0 (SPSS Inc., USA) and Graphpad Prism 9.0 software (Graphpad Prism Inc., USA). The comparison between two or multiple groups were conducted using student's *t*-test or one-way analysis of variance (ANOVA). $p < 0.05$ was set as threshold for statistically significant difference.

3. Results

3.1. Identification of miR-146 b in hUCMSC-derived exosome

The results from flow cytometry showed that the hUCMSCs express high levels of CD44 and CD73 on cell surface and low level of CD45 and CD34 (Fig. S1A), which is consistent with the feature of MSCs. The results from TEM and NTA showed the isolated exosomes with double layer structure and particle diameter distributed from 50 nm to 200 nm (Fig. S1B). Besides, the isolated exosomes contain high levels of CD9, CD63, CD81, and TSG101 and low Calnexin expression (Fig. S1C and Fig. S2). To determine the internalization of exosomes by neurons, the exosomes were labeled by PKH26 and incubated with PC12 cells. As shown in Fig. S1D, the PKH26-labeled exosomes were observed inside the PC12 cells (Fig. S1D). Moreover, the transfection with miR-146 b inhibitors led to decreased exosomal miR-146 b level from hUCMSCs (Fig. S1E), which also decreased the miR-146 b level in PC12 cells that treated with the exosomes (Fig. S1F).

3.2. hUCMSCs-derived exosomal miR-146 b alleviates SCI in rat

We next evaluated the effects of exosomal miR-146 b on spinal cord injury by using a SCI rat model. The spinal cord tissues were isolated for histological analysis and the hind-limb motor function was evaluated by BBB score. We observed that the spinal cord from SCI rats exhibit obvious tissue injury and vacuoles, which were alleviated by hUCMSC-derived exosomes (Fig. 1A). The inhibition of miR-146 b suppressed the protective effects of hUCMSC-derived exosomes on tissue damage (Fig. 1A). As shown in Fig. 1, inhibition of miR-146 b significantly impaired the hUCMSC-derived exosomes-repaired hind-limb motor function. The cell apoptosis in spinal cord tissue was elevated in SCI rats

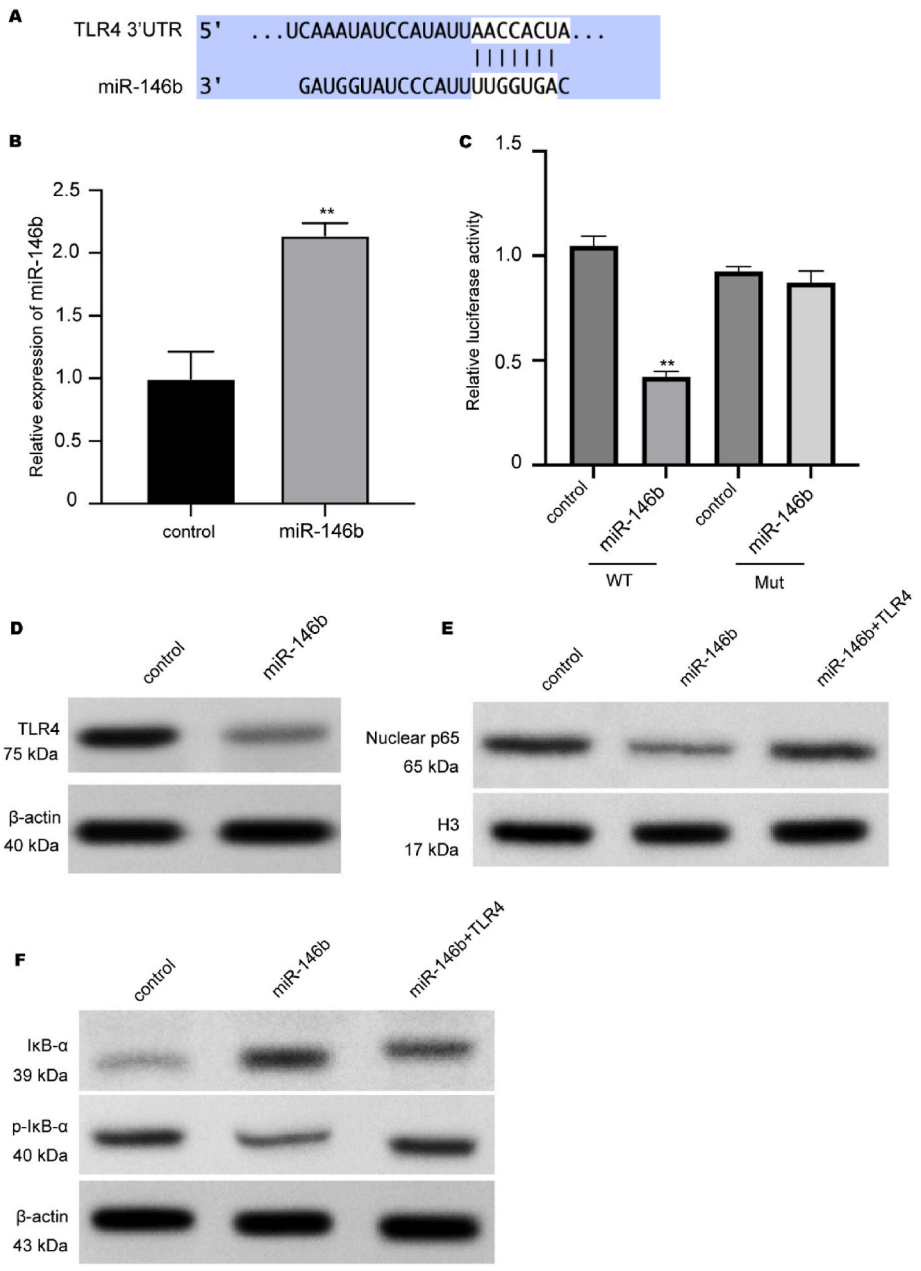

Fig. 3. miR-146 b targets TLR4 to inhibit NF-κB signaling. (A) The prediction of potential binding site between miR-146 b with 3′UTR of TLR4. (B) PC12 cells were transfected with miR-146 b mimics or control mimics for 24 h, then level of miR-146 b was detected by qPCR assay. (C) The luciferase activity of TLR4 reporter gene vectors with wild type (WT) or mutated (MUT) sequences. (D) The protein level of PTEN in PC12 cells after transfection of miR-146 b mimics or control was measured by western blotting. (E and F) PC12 cells were transfected with miR-146 b and TLR4 overexpression vectors, then protein levels of (E) NF-κB in nuclei and (F) phosphorylated IκB and total IκB were measured by western blotting. **p < 0.01.

compared with that in sham group, and hUCMSC-derived exosomes alleviated the cell apoptosis, whereas miR-146 b inhibitors abolished the anti-apoptotic effects of hUCMSC-derived exosomes (Fig. 1C). Results from Fig. 1D–F showed that hUCMSC-derived exosomes enhanced the expression of NeuN and NF200 and decreased the expression of GFAP, indicating the improved neuron proliferation and regeneration and suppressed inflammation.

3.3. hUCMSCs-derived exosomal miR-146 b attenuates PC12 nerve cell injury

Subsequently, we adopted an LPS-stimulated PC12 cell model to mimic SCI-induced inflammatory response in neurons. Treatment with exosomes from hUCMSCs notably improved the cell viability (Fig. 2A) and alleviated the TUNEL-positive cells (Fig. 2B), accompanied by elevated level of Bcl-2 and decreased levels of cleaved caspase-3 (Fig. 2C), whereas inhibition of miR-146 b abolished these effects. Moreover, the elevated expression of GFAP and decreased NF200 caused by LPS was reversed by treatment with exosomes from hUCMSCs, whereas miR-146 b inhibitors abolished the effects of exosomes (Fig. 2C and Fig. S3). Treatment with exosomes from hUCMSCs decreased the release of TNF-α, IL6, and IL-1β by LPS-induced PC12 cells, while inhibition of miR-146 b recovered the expression of these inflammatory cytokines (Fig. 2D–F).

3.4. miR-146 b targets TLR4 to inhibit NF-κB signaling

We next investigated the miR-146 b-regulated target in PC12 cells. Bioinformatic analysis demonstrated that miR-146 potentially target the 3′UTR region of TLR4 mRNA (Fig. 3A). Therefore, we tried to determine whether miR-146 b regulate the expression of TLR4. As shown in Fig. 3B, transfection with miR-146 b notably elevated the miR-146 b level in PC12 cells. The results from luciferase reporter gene assay showed that miR-146 b notably suppressed the luciferase activity of TLR4 reporter gene vectors with wild type (WT) sequences, rather than the mutated (Mut) vectors (Fig. 3C). Moreover, the protein level of TLR4 in PC12

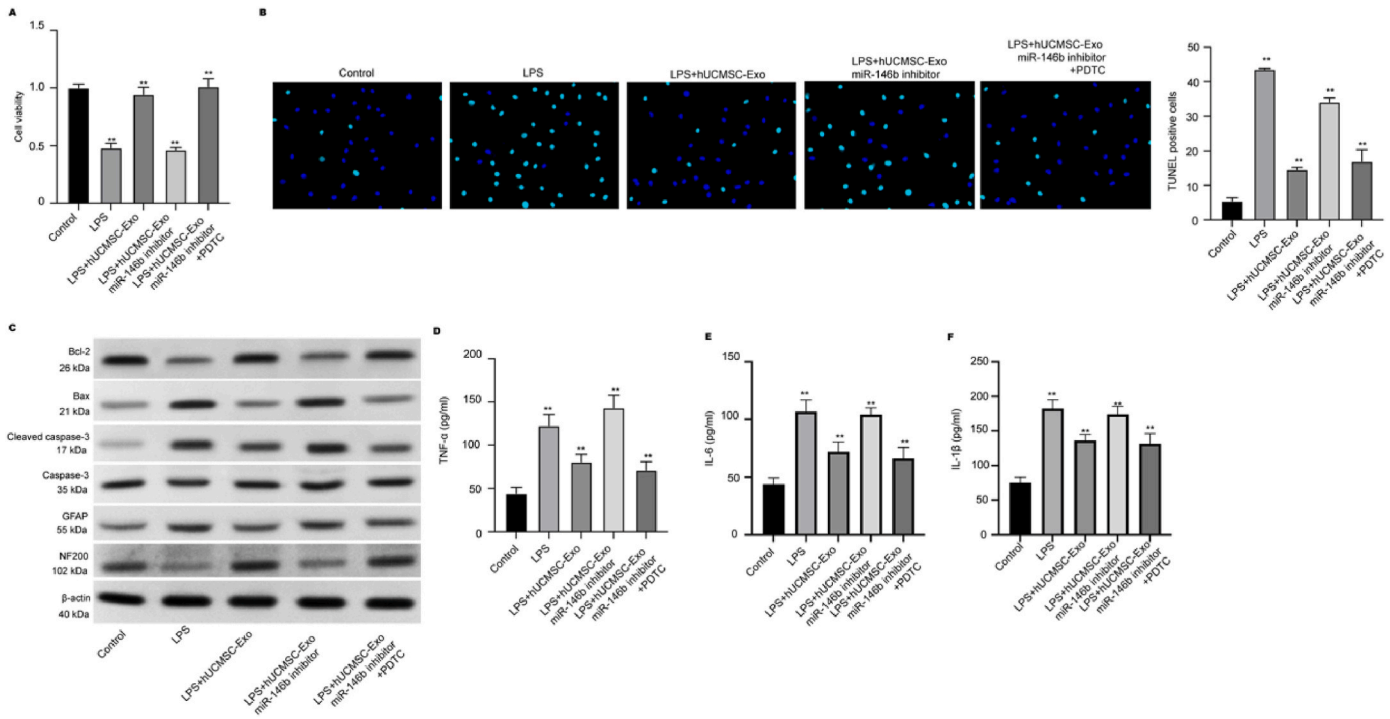

Fig. 4. HUCMSCs-derived exosomal miR-146 b attenuates PC12 nerve cell injury by targeting NF-κB signaling. PC12 cells were stimulated with LPS for 24 h, followed by treatment with hUCMSCs -derived exosomes and NF-κB inhibitor PDTC another 24 h. (A) Cell viability was measured by CCK-8. (B) Cell apoptosis was checked by TUNEL assay. (C) The protein levels of Bcl-2, Bax, cleaved caspase-3, caspase-3, GFAP, and NF200 were detected by western blotting. (D–F) The release of TNF-α, IL-6, and IL-1β were measured by ELISA assay. $^{**}p < 0.01$. Scale bar, 50 μm.

cells was notably downregulated by miR-146 b (Fig. 3D and Fig. S4). The miR-146 b also suppressed the activation of NF-κB signaling, manifested by decreased nuclear p65 level (Fig. 3E and Fig. S4) and suppressed phosphorylation of IκB (Fig. 3F and Fig. S4), whereas overexpression of TLR4 suppressed the effects of miR-146 b. These data indicated that miR-146 b acts as sponge of TLR4 to suppress the NF-κB signaling in PC12 cells.

3.5. hUCMSCs-derived exosomal miR-146 b attenuates PC12 nerve cell injury by targeting NF-κB signaling

To validate the regulatory role of TLR4/NF-κB signaling in the neuronal protective effects of exosomal miR-146 b, we treated the LPS-induced PC12 cells with hUCMSCs-derived exosomes and NF-κB inhibitor (PDTC). The results from CCK-8 and TUNEL assay revealed that inhibition of miR-146 b suppressed the protective effect of hUCMSCs-derived exosomes on LPS-induced neuron death, but administration of PDTC recovered the proliferation (Fig. 4A) and repressed apoptosis (Fig. 4B), as manifested by the changed protein levels of Bcl-2, Bax, cleaved caspase-3, caspase-3, GFAP, and NF200 (Fig. 4C and Fig. S5). Besides, inhibition of NF-κB exhibited similar effects with hUCMSCs-derived exosomes on decreasing the inflammatory cytokine secretion (TNF-α, IL-6, and IL-1β) and reversed the effects of miR-146 b inhibitor (Fig. 4D–F). These results demonstrated that the exosomal miR-146 b protect neuron cell survival via targeting NF-κB signaling.

4. Discussion

SCI is a complex condition that severely threatens human life and health [27]. However, current treatments are not sufficient for prevention and therapy of SCI owing to its complex regulatory mechanisms. MSCs are regarded as promising tool for tissue repairment and regeneration, including the SCI-induced neuron damage [18,28]. Exosomal miRNAs from MSCs are widely reported in various diseases [29]. For example, the exosomal miR-145–5p derived from MSCs could reduce inflammation in spinal cord injury via targeting the nerve growth factor (NGF) and TLR4/NF-κB signaling pathway [29,30]. In this work, we studied the therapeutic mechanism of human umbilical mesenchymal stem cell-derived exosomal miR-146 b in SCI. Our data demonstrated that exosomal miR-146 b effectively alleviated the spinal cord injury in vivo. The miR-146 b has been reported to participate in multiple diseases, such as cancers and inflammatory diseases [31]. For example, miR-146 b improved LPS-induced acute lung injury in a murine model and reduced the production of IL-1β and TNF-α [32]. Liu and colleagues reported that miR-146 b-5p was highly expressed in bone marrow MSCs and alleviated aplastic anemia (AA) through enhancing ubiquitination of PPARγ [33]. Besides, extracellular vesicles that secreted by the M1 macrophages could suppress the migration and invasion of trophoblast by directly targeting the TNF receptor-associated factor 6 (TRAF6) [34].

Subsequently, we verified that miR-146 b suppressed the secretion of inflammatory cytokines, IL-1β, IL-6, and TNF-α, in SCI rat model and LPS-induced PC12 cell model, which is consistent with reported studies. We further explored the targeting relationship of miR-146 b to TLR4 and demonstrated that miR-146 b directly target the 3′UTR of TLR4. TLR4 is a pivotal receptor that regulates the host immune system and induces the expression of inflammatory cytokines via regulating the NF-κB cascade [35]. Studies have revealed that NF-κB could be activated by TLRs to participate in the regulation of inflammatory response and inflammatory microenvironment during SCI [36]. And it is demonstrated that multiple drugs that targeting TLR4 could control the inflammation upon SCI owing to the regulatory axis of TLR4/NF-κB signaling pathway [37]. Furthermore, miR-146 b-5p is reported to enhance the sensitivity of lung cancer cells to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors via targeting the NF-κB signaling pathway [38], which confirms our findings on miR-146 b regulated TLR4/NF-κB signaling pathway. Moreover, a previous study suggested that.

Exosomes derived from miR-146a-5p modified hUCMSC target the neurotoxic astrocytes to facilitate the spinal cord function [39]. In this

work, we used PC12, the adrenal pheochromocytoma cell line as research objective, and demonstrated the exosomal miR-146 b regulates the death and inflammatory response of PC12 cells. However, the miR-146 b could be produced in other cells in brain, such as the neurons, microglia, and astrocytes. What is the effects of miR-146 b that produced by these cells and whether miR-146 b affects the behaviors of primary neurons, microglia, oligodendrocytes and astrocytes remain unknown. Further studies could be conducted to explore the detailed effects of miR-146 b on the neuron system during SCI.

5. Conclusion

In this work, we determined the protective function of hUCMSCs-derived exosomal miR-146 b in SCI. The exosomal miR-146 b significantly alleviated spinal cord injury and inflammatory cytokines via targeting TLR4 to suppress the NF-κB pathway. Our findings may provide novel evidence for application of MSC-derived exosomes in SCI repairment.

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Declaration of competing interest

There are no conflicts of interest.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2023.101497.

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