

Original Article

Neural stem cell-derived exosomes suppress neuronal cell apoptosis by activating autophagy via miR-374-5p/STK-4 axis in spinal cord injury

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Abstract

Objectives: To evaluate the roles of MicroRNAs (miRNAs) enclosed in the neuron-derived exosomes in the recovery of the spinal cord injury (SCI) and the mechanism. **Methods:** The exosomes were isolated from neural stem cells (NSCs) and characterized by transmission electron microscopy (TEM) and NanoSight system (NTA). For *in vivo* experiments, Basso Mouse Scale, beam walking, and inclined plane tests were used to determine the behavioral symptoms of the SCI mice. For *in vitro* experiments, H₂O₂ treated HT22 cells were used to simulate SCI cells and cocultured with exosomes to analyze the cell apoptosis using TUNEL assays and flow cytometry. Apoptosis- and autophagy-related protein expression was detected by western blot and the green fluorescent protein (GFP)-LC3 assay was used to detect the level of autophagy. In addition, luciferase assay was performed to assess the relationship between miR-374-5p and SKT-4. **Results:** Exosomes from NSCs alleviated spinal cord injury by triggering autophagy flux and suppressing apoptosis. Besides, miR-374-5p was highly expressed in these exosomes and was responsible for the decent in injured neural cell apoptosis by activating autophagy flux. The SKT-4 was the target gene regulated by miR-374-5p in this exosomal protective function to SCI cells. **Conclusion:** The elevated level of miR-374-5p in neuronal exosomes could enhance spinal cord injury recovery by activating autophagy.

Keywords: Autophagy, Exosomes, Spinal Cord Injury, Neural Stem Cell, miR-374-5p

Introduction

Spinal cord injury (SCI) is the main cause of chronic motor dysfunction worldwide. In addition to the direct pathological consequences, such as impaired bowel and bladder function and mobility, it can also lead to secondary diseases such as pressure sores, pain, and depression and anxiety¹. Although primary damage leads to direct neuronal death, SCI can still cause serious secondary complications, such as oxidative

stress, inflammatory response, and ischemia, which further aggravate the death of nerve cells, thereby exacerbating the deleterious impact on the individual's life. Although many efforts have been made to reduce these negative effects in order to increase the well-being of SCI patients, the exact molecular pathway of the secondary injury is still elusive. So far, accumulating evidence supports that eliminating those harmful responsive factors induced by SCI will benefit the recovery of patients suffered with SCI²⁻⁴.

Autophagy is an autophagosomal-lysosomal dependent process, degrading and recycling bulk damaged cytosolic components to maintain cellular homeostasis⁵. The autophagosome formation, fusion with the lysosomes, and degradation are the three major steps for autophagy⁶. It has been widely reported that autophagy plays protective roles against SCI, especially in neurons⁷⁻¹⁰, which could suppress neuron death and loss via eliminating toxic cytosolic ingredients¹¹⁻¹³. During the examination of the autophagic levels in neurons during SCI, several biomarkers were widely

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used, such as Beclin1, LC3, and p62. Beclin1 is an important component of pre-autophagosomes¹⁴ and LC3, changed from LC3I to LC3II, is involved in the autophagosome formation¹⁵. Therefore, these two biomarkers are indicators for autophagy initiation. P62, on the other hand, is incorporated into autophagosomes and can be degraded in the lysosomes, which, therefore, is a marker for assessing autophagic degradation¹⁶.

Till now, there is still no effective treatment against SCI. The decrease and the replacement of neural cell loss is a pragmatic goal for restoration of function after SCI. Neural stem cell (NSC) transplantation is now considering as a promising therapeutic strategy for SCI, which could offer renewable source of cells, facilitating neural cell regeneration to confer SCI repair¹⁷. Moreover, nerve cells can secrete exosomes, which are usually small (~100 nm) membrane vesicles, to regulate recipient cell signal transduction, by sending the regulatory molecules, such as miRNAs¹⁸. The role of exosomes secreted by neural stem cells in SCI cells needs further study.

In this study, exosomes isolated from mouse neural stem cells were used to treat mice suffered from SCI, the degree of behavioral recovery was evaluated, and apoptosis and autophagy factors were determined. It was found that exosomal treatment could restore spinal cord injury in mice, and the arachnoid injection of autophagy inhibitor 3-MA inhibited these beneficial effects. To further study the possible underlying mechanism, HT22 cells treated with H₂O₂ were established to simulate nerve cells after SCI *in vitro*. We found that miR-374-5p was highly expressed in exosomes and could promote autophagy and suppress apoptosis in the recipient cells via targeting SKT-4. Our work supplied a novel mechanism of neural stem cells during SCI repair and provided a theoretical basis for the improvement of neural stem cell transplant therapy.

Material and Methods

Animals

A total of 24 healthy adult male C57BL/6 (6–7 weeks) were purchased from the Animal Center of Nanjing University (Nanjing, China). Mice were kept individually in sterile cages under controlled temperature at a 12-h light/dark cycles and could obtained water and food freely. The mice were observed every day for behavioral assessment by two animal behavior specialists.

Spinal cord injury (SCI) model

The SCI model was established according to Allen's method²¹. A total of 24 mice were anesthetized with 4% chloral hydrate (3 ml/kg, Sinopharm group Co., Shanghai, China) before the operation. The mice's backs were opened and the T10 vertebral laminae were exposed and removed gently. To create a moderate compression injury, a 15-g node compressed the exposed spinal cord for 1 min. Operated mice without the compression were denoted as the sham group.

After suturing the muscles and skin, the mice were kept at the electric blanket to recover. The SCI models were established if the mouse's hindlimbs were twisted involuntarily and the tail cramped after the surgery. Cefuroxime sodium was used to prevent infection and bladder evacuation was performed twice a day. The SCI mice were subjected to exosome treatment the next day after surgery^{22,23}. Exosomes were resuspended in 30 µL PBS were used for treatment and PBS only was used as control.

Behavioral function tests

The Basso Mouse Scale (BMS) test²⁴, inclined plane test²⁵, and the Beam walk test²⁶ were used to behavioral evaluation as previously described. All mice were tested at the same time every time at the 1, 3, 7, 14, 21, 28, 35, and 42 days (n=5 per group) post-surgery, 30 min for rest when one test was finished by mice²⁷.

Cell culture and treatment

Mouse neural stem cells were isolated from the forebrains the C57BL/6 mouse without surgery, using the Percoll gradient method²⁸. The cells were cultured in DMEM/F12 medium (Gibco, USA) added 1% N₂ supplement (Gibco, USA), 1 mM L-glutamine (Sigma, USA), 20 ng/ml bFGF (PeproTech, USA), 20 ng/mL EGF (PeproTech, USA), 1% penicillin-streptomycin (Invitrogen, USA) and 50 ng/mL heparin (Sigma, USA). The mouse neuronal cell line HT-22 and HEK-293T cell line were obtained from ATCC (Cellresearch, China) and cultured in Dulbecco's modified Eagles medium (DMEM, Gibco, USA) with 10% FBS (Gibco, USA). To stimulate the SCI condition *in vitro*, cells were treated with 200 µM H₂O₂ for 24 h. 3-MA (2 mM) was added to cells after H₂O₂ treatment. Cells cultured in normoxic conditions were used as the control group. Cells were cultured in a humidified atmosphere with 5% CO₂ at 37°C.

Exosome isolation and identification

Exosomes were prepared from NSCs as previous described²⁹. In brief, the medium was collected and centrifuged at 300×g for 10 min at 4°C. The supernatant was filtered by a 0.22-µm sterile filter (Steritop™ Millipore, MA, USA), transferred, centrifuged at 4000×g for 30 min, transferred, and centrifuged at 10,000×g for 4h at 4°C. The liquid was loaded on the top of a 30% sucrose/D2O cushion and centrifuged at 10,000×g 60 min at 4°C in an Optima L-100 XP Ultracentrifuge (Beckman Coulter). The obtained pellets were resuspended in 200 µL PBS and examined by transmission electron microscope (TEM)³⁰, NanoSight NS300 (NTA, Malvern Instruments, China), and western blot assay for exosome markers: CD63, CD9, and CD81.

RNA extraction and qPCR

Exosomal small RNAs were extracted using a miRcute miRNA isolation kit (Tiangen, China) according to the

Table 1. Primers used for qRT-PCR.

Primers	Forward (5'-3')	Reverse (5'-3')
miR-9	GGACGGACAGCGAGAGGAGGCCAAA	TTTGGCCTCCTCTCGCTGTCCGTCC
miR-103	GAGCAGCATTGTACAG	GTGCAGGGTCCGAGGT
miR-132	UAACAGUCUACAGCCAUGGUCG	UUGUACUACACAAAAGUACUG
miR-10	TACCCTGTAGAACCGAATTTG	GTGCGTGTCTGGAGTC
miR-374-5p	TCAGCGGATATAATACAACCTGC	TATCGTTGTTCTCCACTCCTTAC
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

Table 2. Antibodies used in study.

Antibodies	Source	Provider	Dilution
CD63	Rabbit	Invitrogen (PA5-92370)	1/500
CD9	Rat	Invitrogen (MA1-10309)	1/1000
CD81	Rabbit	Invitrogen (MA5-32333)	1/1000
Calnexin	Mouse	Santa Cruz (sc-23954)	1/1000
Bax	Mouse	Invitrogen (33-6400)	1/500
Bcl-2	Mouse	Invitrogen (MA5-11757)	1/50
caspase-3	Rabbit	Invitrogen (MA5-35335)	1/1000
LC3	Rabbit	Abcam (ab192890)	1/2000
Beclin 1	Rabbit	Abcam (ab207612)	1/2000
p62	Rabbit	Abcam (ab211324)	1/1000
STK-4	Rabbit	ThermoFisher (PA5-22015)	1/600
GAPDH	Mouse	Abcam (ab8245)	1/5000

manufacturer's guideline and cDNA was synthesized using a FastQuant RT Kit (with gDNase) (KR106, Tiangen, China). qRT-PCR was conducted using SYBR green (Toyobo, Japan). The primers of six miRNAs are listed in Table 1. The miRNAs expression levels were analyzed using the $2^{-\Delta\Delta Ct}$ method with U6 as control³¹.

Cell Transfection

Cells were seeded into a 6-well plate with a density of 2×10^5 per well. When the confluence reached ~80%, cells were transfected with pcDNA3.1 vector harboring STK-4, miR-374-5p mimic (forward: 5'-UUAUAUAAUACAACCGCUAAGUG-3'; reverse: 5'-CACUUAUCAGGUUGUAUUAUAA-3'), miR-374-5p inhibitor (5'-CACUUAUCAGGUUGUAUUAUAA-3'), or the related controls using Lipofectamine 3000 (Invitrogen) according to the manufacturers' illustrations.

Flow Cytometry

The flow cytometry analysis was performed according to protocols³². Apoptotic cells were double stained with FITC-Annexin V and propidium iodide (PI) using the FITC Annexin

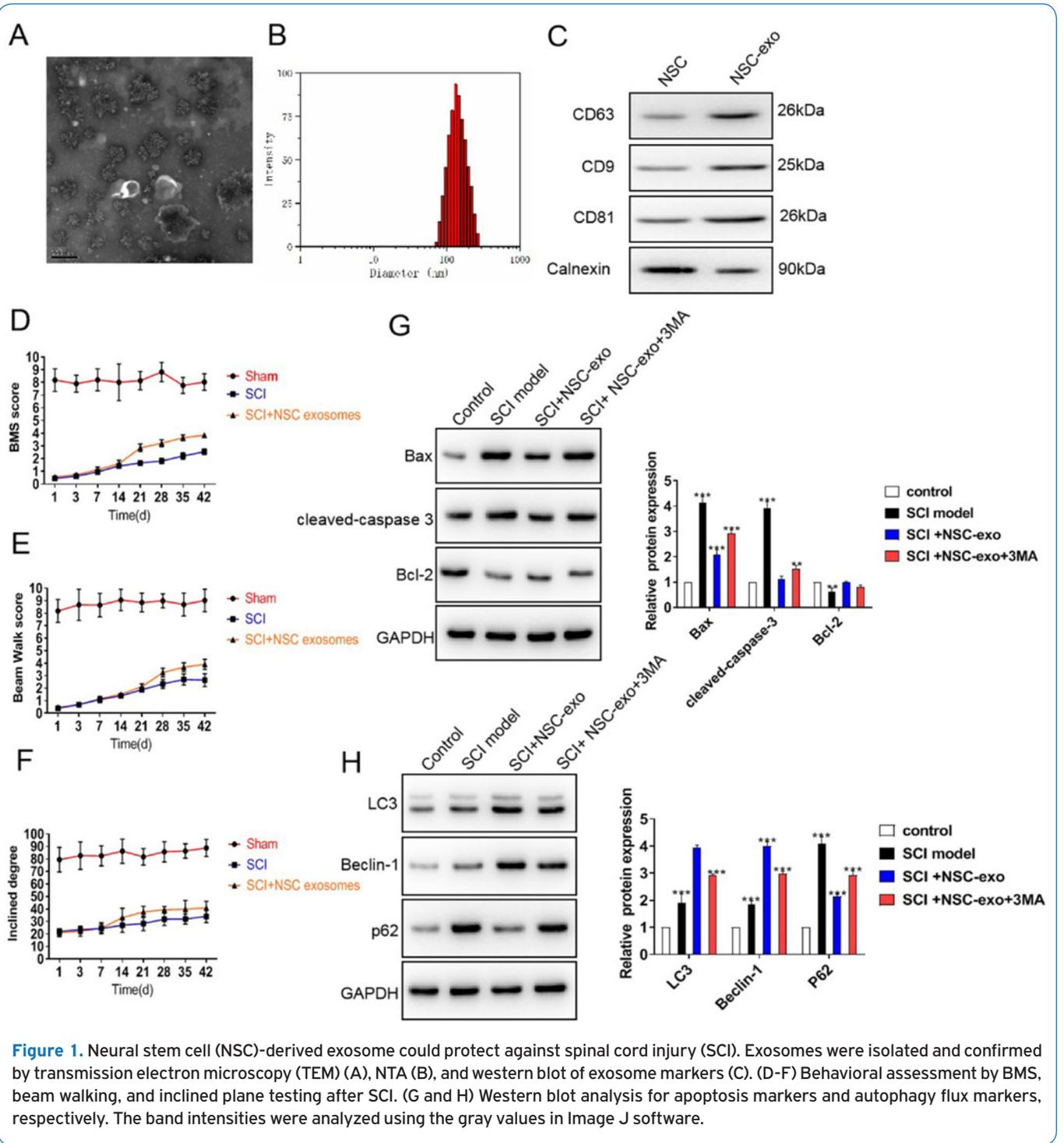
V Apoptosis Detection Kit (ThermoFisher) and the apoptosis rates were analyzed using Flow Cytometry Panel Builder (ThermoFisher).

TUNEL staining

Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) method was employed to examine cell apoptosis. Cells (5×10^7) were fixed in 4% formalin for 10 min. 50-100 μ l of cell suspension was drop on the glass slide and were stained using a TUNEL kit (Promega, USA) according to the manufacturer's instructions.

Immunofluorescence staining

To identify the cultured cells and measure the autophagy levels, immunofluorescence staining was performed as Li et al described³³. The LC3 probe was obtained from Fluoroprobes (China) and the nuclei were denoted with DAPI staining. Cells were observed and analyzed using Olympus microscope (FV 1200) with 30 random vision fields and representative images were shown.



Western blot analysis

Total proteins from the spinal cord tissues or cells were extracted using RIPA Lysis Buffer (ThermoFisher) and were quantified by DC protein assay. Western blot was carried out as previous described³⁴. The primary antibodies were listed in Table 2 and the bands were then developed using enhanced

chemiluminescence chromogenic substrate (GE Healthcare, UK) and analyzed by the Image J software.

Dual-luciferase reporter assay

Vectors containing wide-type (WT) and mutant (MUT) STK-4 3'UTR were co-transfected with miR-374-5p mimic, miR-

374-5p inhibitor or the related controls using Lipofectamine 3000 (Invitrogen). Luciferase assays were performed using the Dual-Luciferase Reporter Assay (Promega).

Statistical analysis

Graphpad Prism 8.0 was used for drawing graphs and statistical analysis. All values were presented as mean \pm SD. One-way ANOVA with Turkey's post-test was used to compare the biochemical indices of different groups and two-tailed Student's *t*-test was used for the comparison between two groups. A *p* value less than 0.05 was considered statistically significant. At least three independent replicants were performed in each test.

Results

Neural stem cell (NSC)-derived exosomes conferred functional improvements in spinal cord injury (SCI) mice

To identify the role of NSC-derived exosomes in the functional recovery of SCI mice, the membrane vesicles secreted from NSCs were isolated and confirmed by transmission electron microscope (TEM) and NanoSight system (NTA), exhibiting a dispersed homogenous population with a in particle size of \sim 105 nm (Figures 1A and 1B). Exosomal marker proteins CD63, CD9, and CD81 could also be detected in these extractions using western blot analysis (Figure 1C). After the subarachnoid injection of the NSC-derived exosomes, the behavioral assessments were performed to evaluate the functional recovery of the injured spinal cord of the SCI mice. According to the Basso Mouse Scale (BMS) test, beam walking test, and inclined plane test, it turned out that SCI mice injected with NSCs-derived exosomes obtained an obvious promotion compared with the SCI group injected with PBS (Figures 1D to 1F). Mice suffered with SCI presenting an increased apoptosis, showing an elevated Bax and Caspase 3 expression and a descent of Bcl-2 level, whereas exosome treatment could ameliorate the SCI induced apoptosis which could be inhibited with the supplement of 3-MA, an autophagy inhibitor. Consistently, exosome treatment in SCI group showed an enhanced autophagy flux compared with the SCI group, reflecting with an increased level of LC3 and beclin-1, and a decreased level of p62, indicating that the suppression effects of the NSCs-derived exosomes on the apoptosis in the SCI mice might be attributed to the stimulation of the autophagy flux in the SCI tissue (Figures 1G and 1H).

NSCs-derived exosome attenuates H₂O₂-induced neuronal apoptosis by activating autophagy in vitro

According to Figure 2A, an increased apoptosis could be observed in HT22 cells treated with the H₂O₂, reflecting an enhanced green fluorescence, whereas the supplement of NSCs exosomes could reduce the cell death rate, whose positive effects could be reversed with the addition of 3-MA, the inhibitor of autophagy. The same trend could be observed

using a flow cytometry assay and the western blot assay of the apoptosis marker Bax, Caspase 3, and Bcl-2 (Figures 2B and 2C). Moreover, the autophagy flux of the exosomes treated injured HT22 cells was also detected by immunofluorescence assay and western blot. It turned out that autophagy was triggered by injury in neural cells which could be enhanced by NSC-exosome treatment (Figures 2D and 2E).

miR-374-5p is essential for the protective effect of the NSCs-exosome against SCI

RT-qPCR showed a significantly elevated expression of miR-374-5p (Figure 3A). According to Fig. 3B, the TUNEL assay exhibited an increased fluorescence intensity in the miR-374-5p KD group compared with the exosome group, indicating an alleviation of the apoptosis inhibition by exosome treatment. Consistently, the flow cytometry assay and the western blot showed similar apoptosis trends (Figures 3C and 3D). The autophagy flux reflected by immunofluorescence and western blot manifested that the miR-374-5p KD group held a decreased autophagy flux in comparison with the exosome group (Figures 3E and 3F).

miR-374-5p activates autophagy of injured neurons through targeting STK-4

The binding site of miR-374-5p and STK-4 was shown in Figure 4A and the interaction between them was examined using a luciferase reporter assay. HEK-293T cells co-transfected with both miR-374-5p and wild type (WT) STK-4 vector, rather than the mutated (MUT) STK-4 vector, showed a decreased luciferase activity. Conversely, an elevated luciferase activity could be observed in cells co-transfected with the WT STK-4 vector and the miR-374-5p inhibitor, indicating an interaction between miR-374-5p and STK-4 (Figure 4B). The transfection of miR-374-5p mimic into the H₂O₂ treated HT22 cells would decrease the protein level of STK-4, which could be retrieved with the co-transfection of the pcDNA3.1 vector containing overexpressed STK-4 (Figure 4C). TUNEL assay displayed a significantly decreased level of fluorescence intensity in injured neurons transfected with miR-374-5p mimic, which was recovered with the co-transfection of overexpressed STK-4 (Figure 4D). Consistently, the flow cytometry assay and the western blot showed similar apoptosis trends (Figures 4E and 4F). Moreover, the immunofluorescence assay and the western blot of the autophagy markers demonstrated that exosomal miR-374-5p/STK-4 axis was responsible for the protective function to the neuron damage via triggering autophagy (Figures 4G and 4H).

Discussion

Spinal cord injury (SCI) usually causes paralysis and reduces people's quality of life. However, due to the complex of SCI, extensive studies are still required to discover more effective treatments. Neural stem cell (NSC) transplantation

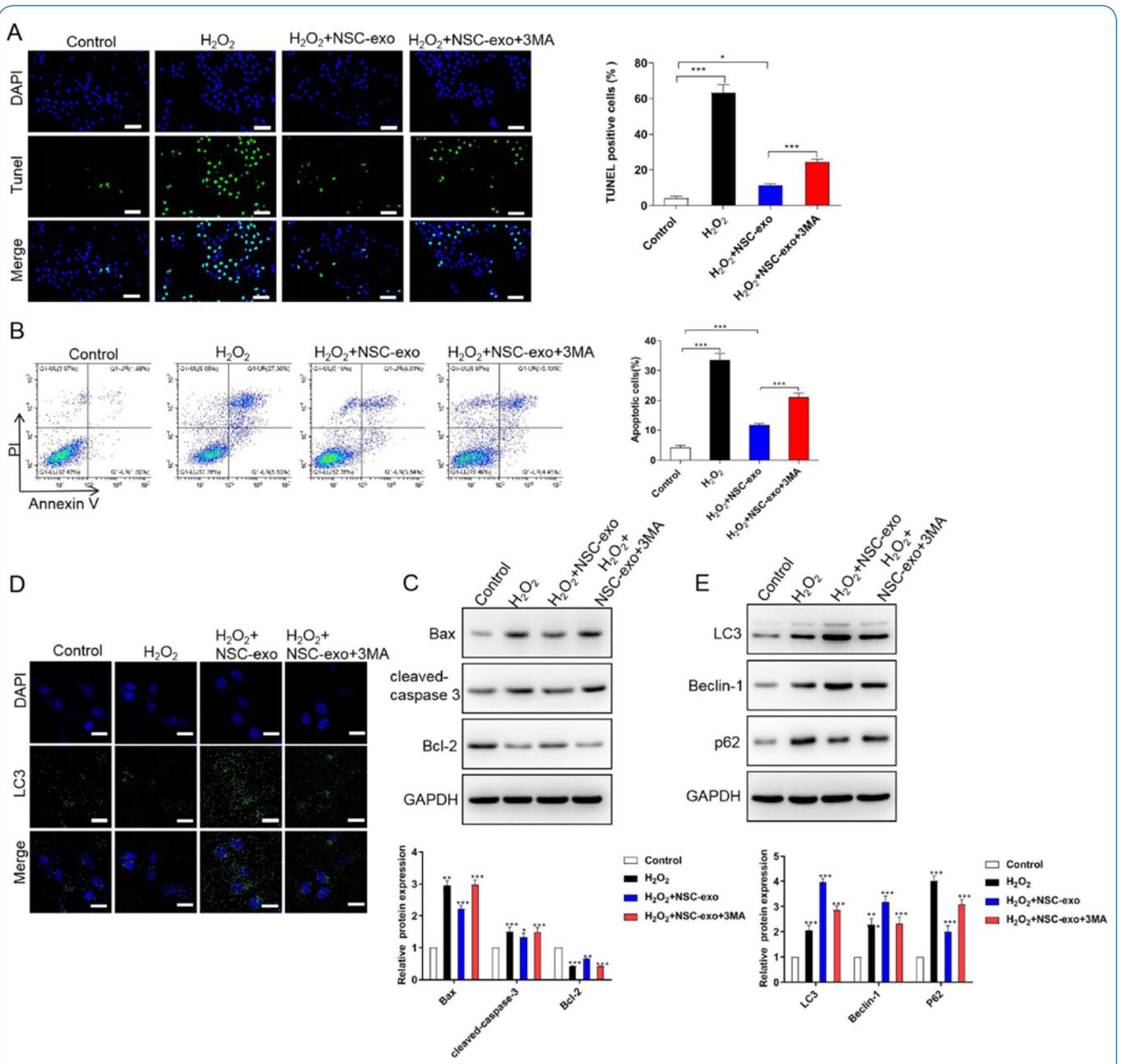


Figure 2. NSC-exosomes attenuates H₂O₂-induced neuronal apoptosis by activating autophagy *in vitro*. Cell apoptosis was examined by TUNEL staining (A), flow cytometry assay (B), and western blot of apoptosis markers (C). The autophagy flux was examined by immunofluorescence staining (D) and western blot of autophagy flux markers (E). The band intensities were analyzed using the gray values in Image J software.

is considered to be one of the most promising therapy for SCI, and the examination of the underlying mechanism would benefit the improvement of such therapy. In this work, we focused on the function of NSC derived exosomes on SCI recovery. We reported that NSC-exosomes could trigger autophagy and suppress apoptosis to benefit SCI repair *in vitro* and *in vivo* via miR-374-5p/STK-4 axis.

Bcl-2 is an mitochondria integral membrane protein, whose overexpression inhibit cell apoptosis³⁵, whereas Bax, a Bcl-2 related protein promotes cell apoptosis³⁶. Caspase-3 also acts as a death activator³⁷. In the present study, SCI mice presented an increased apoptosis, showing an elevated Bax and Caspase 3 expression and a descent of Bcl-2 level, whereas exosomes derived from NSCs ameliorated the

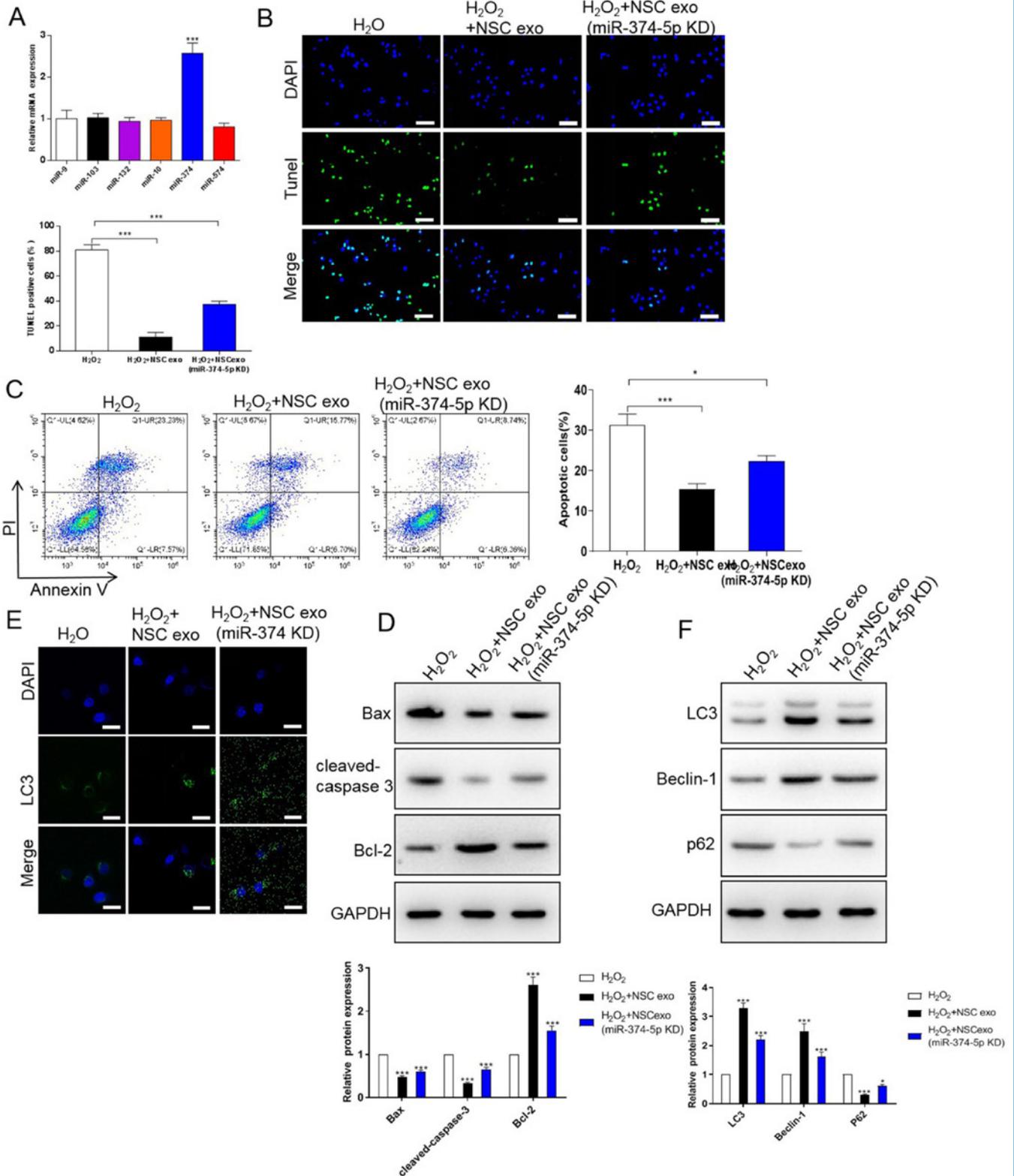


Figure 3. MiR-374-5p is essential for the protective effect of NSC-exosome on SCI. (A) Identification of exosome miRNA expression levels. Cell apoptosis was examined by TUNEL staining (B), flow cytometry assay (C), and western blot of apoptosis markers (D). The autophagy flux was examined by immunofluorescence staining (E) and western blot of autophagy flux markers (F). The band intensities were analyzed using the gray values in Image J software.

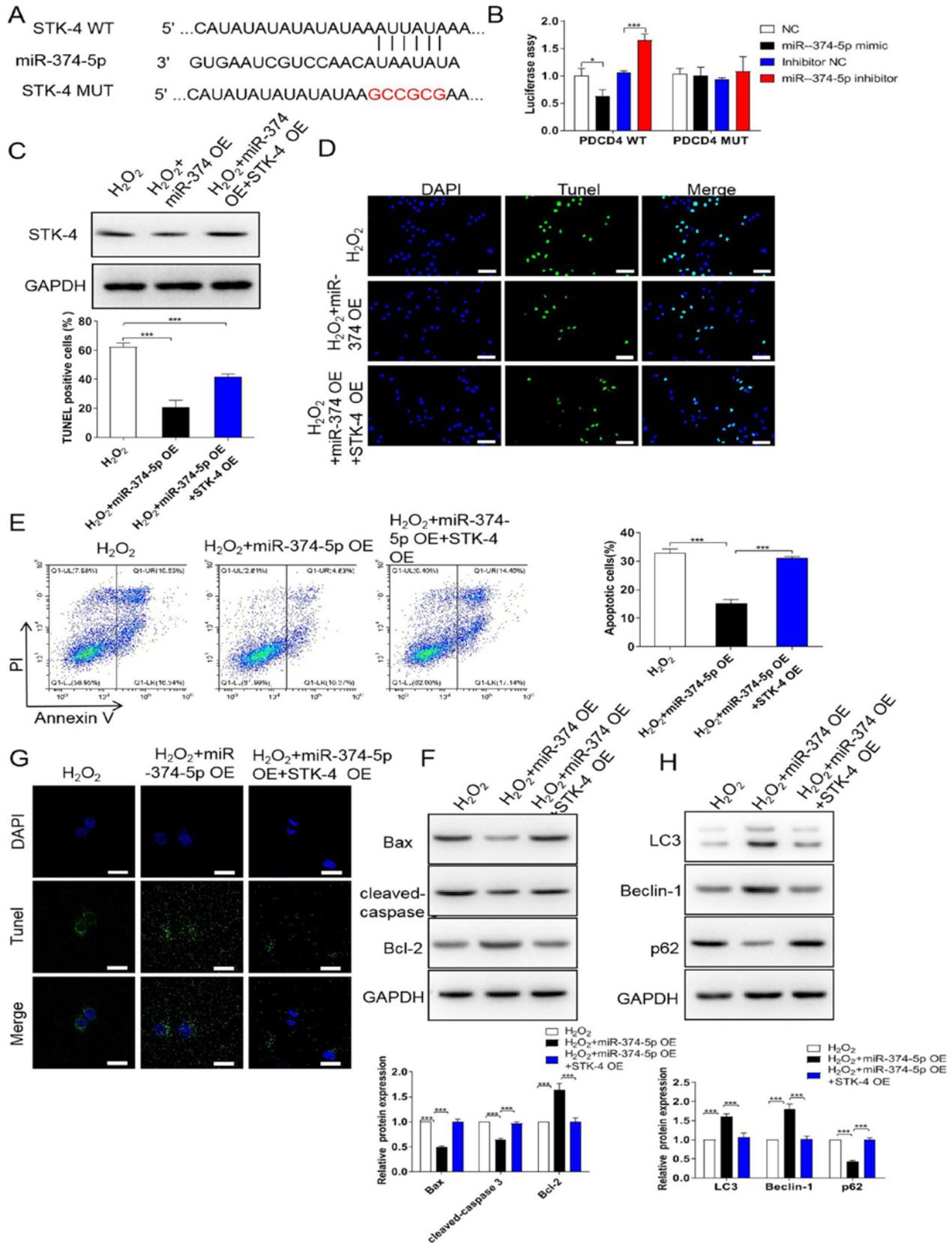


Figure 4. MiR-374-5p activates autophagy of injured neurons through directly targeting STK-4. (A) Bioinformatics prediction of binding sites between miR-374-5p and the 3'UTR of the STK-4 mRNA. (B) The interaction of miR-374-5p and the 3'UTR of the STK-4 mRNA was examined by dual-luciferase reporter assay. (C) The expression level of STK-4 was detected by western blot. Cell apoptosis was examined by TUNEL staining (D), flow cytometry assay (E), and western blot of apoptosis markers (F). The autophagy flux was examined by immunofluorescence staining (G) and western blot of autophagy flux markers (H). The band intensities were analyzed using the gray values in Image J software.

SCI induced apoptosis which could be inhibited with the supplement of 3-MA, an autophagy inhibitor.

Autophagy has been reported to play important roles in SCI³⁸. The autophagy flux was also determined with the autophagic markers, which were LC3, proteins composing the autophagosome membranes³⁹, Beclin-1, an essential protein at the beginning of autophagy⁴⁰, and p62, proteins facilitating the degradation of autophagosomes⁴¹. Whether the enhanced autophagy level is beneficial or detrimental to neural cell maintenance during the SCI repair depends on the context. For instance, after SCI, the supplement of rapamycin, an autophagic agonist, could enhance the functional recovery of the neural cells, whereas 3-MA, an autophagy inhibitor, aggregated the neuron damage⁴, indicating that the activation of autophagy could depress neuronal cell death and loss. Conversely, Kanno et al. found that autophagic cell death, featuring by TUNEL-positive and highly expressed LC3, occurred in SCI model⁴², which might lead to beneficial outcomes⁹. Therefore, the protective mechanism of the autophagy in SCI needed to be further investigated. In our work, we found that exosomes derived from NSCs could diminish cell apoptosis by stimulating autophagy process in SCI model *in vitro* or *in vivo*, suggesting a protective function of autophagy process in neurons recovery.

Many miRNAs have been reported to be expressed in the NSC cells and can be enclosed in the exosomes, and some of which were believed to exert important impact on neural cell damages of diverse kinds. Based on the published articles, we profiled miR-9⁴³, miR-103⁴³, miR-132⁴⁴, miR-10⁴⁵, miR-374⁴⁶ and miR-574¹⁸ in the extracted exosomes using RT-qPCR and found a significantly elevated expression of miR-374-5p. miR-374-5p, located at the X-chromosome, has been reported to play an indispensable regulatory role in neural system diseases, such as epilepsy, neurodegeneration, and hypoxic-ischemic encephalopathy⁴⁷. To verify whether miR-374-5p was responsible for the protection of the injured neural cells, we co-cultured the H₂O₂ treated HT22 cells with the exosomes obtained from NSCs transfected with the miR-374-5p inhibitor (denoted as the miR-374-5p KD group). In this work, TUNEL assay, flow cytometry assay, and western blot assay showed that the silencing of miR-374-5p increased injured neural cell death by inhibiting autophagic level. All these data indicated that miR-374-5p enclosed in the NSC-exosomes might confer beneficial function in neural cell damage via activating autophagy.

We then digging the regulatory mechanisms of the exosomal miR-374-5p mediated neural cell protection. The target genes of this miRNA were predicted using TargetScan 7.2 (http://www.targetscan.org/vert_72/). Bioinformatical analysis and luciferase assay showed that serine/threonine kinase 4 (STK-4) was a direct target of miR-374-5p. STK-4, also widely known as MST-1 in mammals, was widely reported to participate in cell death regulation⁴⁸⁻⁵⁰. In our work, we found that STK-4 was down regulated by highly expressed miR-374-5p from NSC-exosomes, which could improve SCI cell viability by triggering autophagic process, which is in consistent with works of Zhang et al⁵¹.

In sum, our work found an increased level of miR-374-5p in NSC-exosomes, which exerted a protective function on spinal cord injury by activating autophagy via targeting STK-4, providing novel evidence for the mechanism underlying the NSC transplant therapy for SCI.

Ethics approval

All applicable institutional and/or national guidelines for the care and use of animals were followed. The animal experimental protocol has been approved by The Second Affiliated Hospital of Xi'an Medical University (permit number: X2Y202214). Also, we have followed ARRIVE guidelines^{19,20}.

Authors' contributions

LZ conceived and designed the study, and drafted the manuscript. PH collected, analyzed and interpreted the experimental data. LZ and PH revised the manuscript for important intellectual content. Both authors read and approved the final manuscript.

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