Neural stem cell-derived exosomes protect spinal cord injury by the transfer of miR-31-5p

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Abstract

Traumatic spinal cord injury (SCI) is a catastrophic damage that causes changes in its motor function permanently. Reactive astrocytes is a pathological feature of spinal cord injury (SCI). Exosomes take part in the transportation of miRNAs and play an a novel platform for intercellular communication in the central nervous system (CNS). However, the effect of miRNAs in Neuronal stem cells (NSCs) derived exosomes in SCI was unknown. in vivo SCI model and in vitro experiments were performed to investigate the effects and mechanisms of exosomes. NSCs-derived exosomes promoted motor function recovery by shifting astrocytes from the A1 to A2 phenotype. microarray analysis of miRNA showed that miR-31-5p was the most enriched in NSCs-derived exosomes. Bioinformatics, RIP, and luciferase activity predicted IL34 was the target downstream gene of miR-31-5p. Western bloting examined IL34/STAT3 signaling pathway involved in modulating atrocities by the exosomal miR-31-5p. Rescue experiments evaluate that exosomal miR-31-5p shifting astrocytes A1 to A2 phenotype by inhibiting IL34/STAT3 signaling cascades, and promoted motor function recovery in mice after SCI.

Introduction

Spinal cord injury (SCI) is a damage with an external physical impact, and causes changes in its function permanently. SCI have devastating physical, social, and vocational consequences for patients. In the complex injury cascade, organization and structural of the spinal cord have changed, including the formation of glial scar, cystic cavities, and axonal regrowth. This poor intrinsic recovery potential causes permanent neurological deficits[1].

Astrocytes are abundant and perform the key functions in neurotrophic support[2]. Resting astrocytes are stimulated for conversion to reactive functional phenotypes A1 and A2. A1 astrocytes are classically induced by activated neuroinflammatory microglia. Through secretion neurotoxins, resulting in synaptic destruction and neuron growth inhibition[3]. A2 astrocytes are considered as neuroprotective phenotypes after injury, and regulate neuroprotective factors which promote synaptic repair and growth[4].

Neural stem cells could self-renewing, generating neurons and glia during central nervous system (CNS) development and seems to be a promising therapy, because of the potential to exert multiple reparative actions within CNS via cell replacement and paracrine effects[5]. NSCs transplantation can promote the recovery of neurological function after SCI[6]. However, stem cells grafts integrate into sites of target tissues remains challenging. Transplanted stem cells may have different interactions with the microenvironment of the target site, such as tumorigenicity, immunogenicity, and heterogeneity. It restrict the clinical application of NSCs transplantation for the treatment of SCI[7].

Exosomes (30–200 nm) are secreted by cells as a novel platform for intercellular communication in CNS[8]. It also plays a vital role in the communication between neuron and glial cells. Exosomes can transfer substances through the blood-brain barrier (BBB).Contributing to the development of CNS and

the maintenance of homeostasis[9], exosomes have been proven to have a repairing effect on neurological diseases and are potential treatment tools[10].

MicroRNAs(miRNAs) was a small non-coding RNAs, which mediate post-transcriptional gene silencing by binding to target mRNAs[11]. miRNAs can be exported and imported by cells through exosomes. Exosomes mediate a new miRNAs mechanism in cell-to-cell communication[12]. Exosomes in CNS perform their biological functions by delivering specific miRNAs, from normal development and homeostasis to disease and regeneration[13]. Exosomes derived from NSCs transport a shipment of miRNAs. It is important to use exosomes derived from NSCs for the treatment of neurological disorders. But few studies have been published to date.

We have attempted to improve exosomal bioactivity through the regulation of miRNAs. Our microarray analysis of miRNA showed that miR-31-5p was the most enriched miRNA in NSCs-derived exosome. It could shift astrocytes phenotype from A1 to A2 by regulating IL34/STAT3 signaling cascade to promoted motor function recovery after SCI in mice.

Materials And Methods

Isolation and identification of NSCs-derived exosome.

Neural stem cells were obtained from 13.5 day-old fetal mice and cultured with medium. The medium was collected and centrifuged. Following centrifugation, filtering the cellular supernatant. The filtered supernatant was centrifuged at 4000×g, and centrifuged at 100, 000g in an optima Ultracentrifuge (Beckman Coulter) to purify the exosomes. The extracted exosomes were utilized for downstream experiments. The protein concentration of exosomes was quantified using a bicinchoninic acid assay (BCA; Thermo Fisher Scientific, MA). The characterization of exosomes were performed by Nanosight LM10 System (Nanosight Ltd., Novato, CA), transmission electron microscope (TEM; Tecnai 12; Philips, The Netherlands), and Western blotting.

Primary Astrocyte And Microglial Cultures And Treatment

Primary astrocytes were removed from the mouse brain on day one and cut into pieces to trypsin-EDTA (Thermo Fisher Scientific, MA, USA). The digested tissue was centrifuged (300×g, 5 min) and resuspended. The last monocellular suspension was cultured in T75 to obtain the combined cultures of glial cells. After 3 days, the combined cultures of glial cells were shaken (200 rpm, 6 h) to extract microglia and oligodendrocyte precursor cells (OPCs). The remaining astrocytes were used for experiments.

Primary mixed glial cells were prepared, and microglia were separated (200 rpm, 2 h) after 14 days. LPS (1 μg/mL) was used to treated with microglia for 24 h to obtain activated microglia. LPS- treated microglia conditioned medium was collected as the MCM.

Exosome Uptake By Astrocyte

Exosomes were incubated with Dil (Molecular Probes, USA) solution. Ultracentrifugation was used(100,000×g, 1h, 4°C)to remove excessive dye from labeled exosomes. Astrocytes were co-cultured with these Dil-labeled exosomes for 24h.

Lentivirus production and vector constructs,

miR-31-5p-mimic (miR-31-5p^{OE}), miR-31-5p-inhibitor (miR-31-5p^{KD}), and the overexpression and shRNA vectors of IL34 (IL34 and shIL34) were constructed by lentiviral vectors (Gene Pharma, Shanghai, China). The negative control was established by empty lentivirus (miR-NC^{KD}, miR-NC^{OE}, shNC, and vector).

Fam-labeled Mir-31-5p Transferable Detection

5'-carboxyfluorescein (FAM)-labeled was used to transfer NSCs negative controls, miR-31-5p mimics, and miR-31-5p inhibitor (Gene Pharma). Then, exosomes of different groups were added to astrocytes. Astrocytes are sequentially incubated with formaldehyde (PFA), Triton and antibodies (anti-GFAP). Confocal microscope were used to acquire images and observe signaling intensity.

Mirna Microarray Assay

We treated astrocytes with exosomes and pbs(n = 3), and carried out an microRNA array 21.0 (Agilent-Mouse, 8*60 K, Design ID:070155) (OE Biotech, Shanghai, China). The Affymetrix miRNA 4.0 platform was employed for microarray analysis. miRNAs expression were identified using a fold change of ≥ 1.5 set.

Sci Model

The animal protocols were approved by the Animal Committee Nanjing Medical University. The SCI model was established in C57BL/6 mice (8 week-old), mice were anesthetized by isoflurane inhalation, and then exposing the spinal cord at T8 by laminectomy. An impactor was used to induce SCI by dropping a rod (5 g, 6.5 cm) onto the spinal cord (RWD, CA, USA). The bladders were voided 3 times per day.

Immunofluorescence Staining

The hearts of mouse were sequentially perfused with saline and FPA. The injured spinal cords were fixed in PFA, and then dehydrated in sucrose solutions. For immunofluorescence staining, the frozen sections were incubated with primary antibodies (anti-GFAP, anti-C3, anti-S100a10, and anti-NF200), and then added secondary antibodies (Alexa Fluor 594, Alexa Fluor 488). DAPI (Invitrogen, CA, USA) was used for

counterstaining. The images were gained by LSM800 con-focal microscope (Zeiss, Heidenheim, Germany).

Luciferase Reporter Assay

The 3'-UTR of IL34 mRNA, the wild-type (WT), or mutated (MUT) miR-31-5p binding sequence were synthesized (GeneScript, Nanjing, China). These sequences corresponding were cloned into the pGL3 luciferase control reporter vector to create the IL34 3'-UTR reporter constructs (pGL3-WT-IL34 and pGL3-MUT-IL34). Astrocytes transfected with miR-31-5p^{OE} or negative control were ransfected with pGL3-WT-IL34, pGL3-MUT-IL34 3'-UTR.

Functional Behavioral Analysis

The BMS score was carried out to calculate neurological function for locomotion at 1, 3, 7, 14, and 28 days post-injury. Scoring ranged from 0 (complete paraplegia) to 9 (normal function).

Footprint analysis was carried out. The blue forelimbs and red hind limbs were dipped into dyes. Calculate the stride length of mice running at a constant speed.

MEPs was calculate by electromyography. Placing a stimulating electrode at the end of the rostral of the exposed spinal cord, the reference electrode was inserted at the distal tendon of the hind limb muscle, the recording electrode was placed at the flexor of the biceps femoris, and under the skin placed ground electrode. Appling a single square wave stimulus (0.5 mA, 0.5 ms, 1Hz).

Western Blotting

Total protein were extracted from spinal cord tissues and cells, and treated with RIPA lysis and extraction buffers (Key Gen, Nanjing, China). Equal amounts of protein were separated by SDS-PAGE, and transferred to PVDF membranes (Millipore, Burlington, MA). After blocked with BSA, the membrane was incubated with primary antibodies overnight. Membranes were incubated with secondary antibody. Immunolabeled bands were visualized using enhanced chemiluminescent reagent (Millipore). ImageJ (National Institutes of Health, MD, USA) was performed to Quantify band intensity.

Rna Isolation And Qpcr

Total RNA was extracted by the trizol reagent (Invitrogen, NY, USA) according to the manufacturer's protocol, and the cDNA was amplified by the PrimeScript™ RT Master Mix for qPCR (Takara, Dalian, China). The qPCR was performed by a real-time T100 PCR system (BIO RAD, CA, USA) using AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China). Primer sequences are listed in Supplemental S1.

Staistical Analysis

Experiments were carried out at least three independent biological replicates. Data were described as mean ± standard. Prism 9.2 (GraphPad, CA, USA) was used to manipulate statistical analyses. For two-group comparisons, the unpaired two-tailed Student's t-test was used. For more than two-group comparisons, one-way or two-way ANOVA was used. $p < 0.05$ was considered to be significant statistically.

Results

Characterization of NSCs exosomes

NSCs proliferated into neurospheres, and nestin was detected as the stem cell markers (Figure S1). Exosomes were identified by TEM, NTA, and western blotting. TEM revealed typical nano particles structures, and NTA revealed the size distribution between 30 and 200nm (Fig. 1a, b). Western blotting revealed CD81,CD9, CD63, and calnexin of exosomal surface markers (Fig. 1c). The uptake of Dil-labeled exosomes by astrocytes was observed (Fig. 1d). These results verified exosomes were isolated from NSCs cultures.

Nscs-derived Exosomes Promote Functional Recovery Following Traumatic Sci

To evaluate motor function after SCI. BMS scores, gait analysis, and electrophysiology analysis were applied after treated with saline or exosomes. SCI was observed in two groups on postoperative day 1. At day 28 post-injury, the dexterity of hindlimbs for mice in Exos group was significantly higher than that treated with saline (Fig. 1e). Gait analysis was also used to assess the locomotor activity of hind paws, and the stride length of footprints was significantly shorter in saline group than that treated with exosomes (Fig. 1f). Moreover, In electrophysiology analysis, MEP amplitudes in the Exos group were higher than that in saline groups (Fig. 1g). The density of axons in the injured spinal cord was examined by immunostaining the neurofilament (NF200) to evaluate the neuronal and axonal damage at day 28 post-injury. NF200 was a protein in CNS and qualified as a potential surrogate marker of damage to the axon[14]. GFAP was used to indicate the injury area. The result showed that the intensity of NF200 was much lower in the saline group (Fig. 1h). These experiments suggested that NSCs-derived exosomes could promoted motor function recovery and axonal grown after SCI.

Exosomes Promoted Astrocytes Phenotype From A1 To A2 In Vivo

Activated microglia secreted pro-inflammatory cytokines which induce astrocytes of A1 phenotype after SCI. A1 astrocytes lead to apoptosis of neurons though neurotoxins release. As astrocytes have two different phenotypes, A2 astrocytes regulate neuroprotective factors which promote synaptic repair and

growth. We evaluated whether exosomes could shift astrocyte A1/A2 phenotypes to promote motor function recovery after SCI. The gene expression of A1 (H2d1 and Serping1) and A2 (Ptx3 and S1pr3) were analyzed by qRT-PCR. The gene expression of Ptx3 and S1pr3 was significantly increased in the Exos groups, and that of H2d1 and Serping1 was decreased compared with the saline group (Fig. 2a). Next, representative A1-assocaited C3 and A2-associated S100a10 markers for immunofluorescent staining with GFAP were texted to evaluated the polarization of astrocytes. At day 28 post-injury, C3-positive astrocytes had a marked decrease and S100a10 in astrocytes had a higher level in the Exos groups compared with the saline group in the lesion areas.(Fig. 2c). Furthermore, western blotting further confirmed these results (Fig. 2b). In summary, these results established that exosomes had a notably effect on shifting astrocytes from A1 to A2 phenotype.

Exosomes Shifted Astrocytes From A1 To A2 Phenotype In Primary Astrocytes In Vitro

To investigate activated astrocytes in vitro, we treated astrocytes with microglial conditioned medium for 24h. The gene expression of A1-related (H2d1 and Serping1) and A2-related (Ptx3 and S1pr3) were assessed in different groups. Exosomes decrease the expression level of A1-related genes and increase A2-related genes (Fig. 2d). Immunofluorescence showed that exosomes significantly affected the intensity of C3 and S100a10 in astrocytes (Fig. 2f). Western blotting further confirmed these results, which was similar to in vivo (Fig. 2e). These results revealed that exosomes was important in shifting astrocytes from A1 to A2 phenotype in vitro, and consistent with in vivo experiments.

Mir-31-5p Is Upregulated In Exosomes And Transferred To Astrocytes By Exosomes

The analyses of data from our experiments have shown that NSCs derived exosomes promoted motor function recovery and shifted astrocytes polarization from A1 to A2. As miRNAs play an important functional components of exosomes. Exosomes perform their biological functions by delivering specific miRNAs in CNS. We isolated RNA from astrocytes which were treated with PBS and exosomes, and performed a microarray of miRNAs (Fig. 3a). From these miRNA profiling data, miR-31-5p, miR-132-3p, miR-421, miR-195a, and miR-22-3p were the top five upregulated miRNAs. We used qRT-PCR to further text their expression. According to the microarray and qRT-PCR results, miR-31-5p have shown the most significantly increased in astrocytes (Fig. 3b). Furthermore, We constructed the negative control (miR-NC^{OE} and miR-NC^{KD}), miR-31-5p overexpression (miR-31-$5p^{OE}$), and miR-31-5p knockdown (miR-31-$5p^{KD}$) NSCs. The qRT-PCR was used to text transfection efficiency (Fig. 3c). Next, we isolated exosomes from NSCs (miR-31-$5p^{KD}$-Exos, miR-NC^{KD}-Exos, miR-31-$5p^{OE}$-Exos and miR-NC^{OE}-Exos). In comparison with the miR-NC^{KD}-Exos and miR-NC^{OE}-Exos, the expression of miR-31-5p in miR-31-$5p^{KD}$-Exos has a significant decrease, and an increase miR-31-5p expression in miR-31-$5p^{OE}$-Exos (Fig. 3d). In target astrocytes, when compared to the miR-NC^{OE}-Exos and miR-NC^{KD}-Exos group, miR-31-$5p^{OE}$-Exos group exhibited an

increased expression, and miR-31-5p^{KD}-Exos group showed a decreased expression (Fig. 3e). The immunofluorescence data demonstrated the FAM- labeled miR-31-5p in exosomes was internalized into astrocytes. FAM-labeled exosomal miR-31-5p intensity was significantly lower in miR-31-5p^{KD}-Exos group. Meanwhile, administration of miR-31-5p^{OE}-Exos lead to an increased intensity in astrocytes(Fig. 3f). It was similar to our qRT-PCR results. Overall, summarize the results demonstrated that NSC-derived exosomal miR-31-5p could be transferred to astrocytes.

Exosomes promoted motor function recovery and shifted astrocytes from A1 to A2 phenotype through delivering miR-31-5p.

Firstly, To investigate exosomal miR-31-5p in the development of motor function recovery after SCI, BMS score was carried out as functional behavioral experiments. We found that miR-31-5p^{OE}-Exos could promote functional recovery. In contrast, miR-31-5p^{KD}-Exos canceled the functional effects with exosomes (Fig. 4a,b). The result have shown that exosomes elevated functional behavioral recovery through delivering miR-31-5p. Next, we verified the role of miR-31-5p in astrocytes polarization. A1 and A2 marker levels were detected by qRT-PCR. The results showed that miR-31-5p^{OE}-Exos facilitated the polarization of astrocytes from A1 to A2 and miR-31-5p^{KD}-Exos accounted for the opposite effects following SCI (Fig. 4c,d). Furthermore, the immunofluorescence and western blotting confirmed the qRT-PCR results (Fig. 4e-f).

We carried out in vitro experiments in astrocytes to further evaluate exosomal miR-31-5p. The qRT-PCR results reported that the polarization of astrocytes from A1 to A2 in miR-31-5p^{OE}-Exos group, and it were opposite for administration of miR-31-5p^{KD}-Exos (Fig. 5a,b). Moreover, immunofluorescence and western blotting analysis confirmed these findings (Fig. 5c,d). From all these results, we could concluded that NSC-derived exosomes shifted astrocytes polarization from A1 to A2 phenotype by delivering miR-31-5p.

Exosomal Mir-31-5p Regulates Il34 By Targeting 3'-utr

To explored the mechanism of action for exosomal miR-31-5p. According to the online data-base of miRNA targets, we searched the predicted mRNA targets for miR-31-5p. IL34, STK40 and EBF3 were predicated (Fig. 6a). We then examined the expression levels of target genes by qRT-PCR after administration of miR-NC-Exos and miR-31-5p^{OE}-Exos in astrocytes. We found that IL34 may be the potential target. Moreover, It has been demonstrated that deficiency of IL-34 were protected from synapse loss during virus-induced memory impairment. The WT and MUT 3'-UTR of IL-34 were constructed to verify IL-34 3'UTR was target for miR-31-5p (Fig. 6b). Luciferase reporter assay was used to analyze transfected astrocytes. When overexpressed miR-31-5p was co-transfected with the IL34 WT luciferase construct, the relative luciferase activity was decreased (Fig. 6c). RNA-ChIP analysis was used to detect

the abundance of IL34 mRNA in Ago2/RNA-induced silencing complex (RISC) after miR-31-5p overexpression (Fig. 6d-f). The level of IL34 incorporated into RISC was observed to be enriched in miR-31-5p overexpressing cells. The results of qRT-PCR and Western blotting showed that the overexpression of miR-31-5p reduced the expression level of IL34 mRNA and protein, and the knockdown of miR-31-5p increased the expression level of IL34 (Fig. 6g, h). These results confirmed that IL34 was the target gene of miR-31-5p.

Exosomal Mir-31-5p Regulates Astrocytes A1/a2 Polarization By Targeting Il34

To further evaluate the effect of exosomal miR-31-5p on the regulation of IL34-mediated astrocyte phenotype. Lentivirus and shRNA were used to overexpressed and silenced endogenous IL34 expression in astrocytes. qRT-PCR was used to test the astrocytes A1-and A2-related genes (Fig. 7a,c). Overexpression of IL34 inhibit A1 towards A2 polarization, knockdown of IL34 exhibited the opposite effect. We next studied the offset effect of IL 34 on miR-31-5p^{OE}-Exos. The results showed that IL34 overexpression inhibit A1 towards A2 polarization induced by miR-31-5p^{OE}-Exos. The unfavorable effects of miR-31-5p^{KD}-Exos were counteracted by IL34 downregulation in astrocytes. Results of western blot (Fig. 7b,d) and immunofluorescence (Figures S2) demonstrated that knockdown of IL34 could reverse astrocytes phenotype induced by miR-31-5p^{KD}-Exos and promote A1 to A2 polarization. Similarly, the beneficial effects of miR-31-5p^{OE}-Exos were counteracted by overexpression of IL34. The above experiments proved that miR-31-5p of exosome regulate astrocytes from A1 to A2 phenotype by targeting IL34.

Mir-31-5p Of Exosome Regulates A1/a2 Astrocytes Polarization Through Stat3 Signaling Cascades

STAT3 involved in the activation of A1 astrocytes in SCI, and Inhibition of STAT3 could switch A1 to A2 astrocytes phenotypic[15]. Moreover, it has reported that there is interference between IL-34 and STAT3 signaling pathways[16, 17]. Therefore, we hypothesize that exosomes have an effect on astrocytes A1/A2 polarization via the IL34/STAT3 signaling pathways. Western blotting was used to evaluate the expression of major members of the STAT3 pathway (Fig. 7e,f). When administration of miR-31-5p^{OE}-Exos, the expression of S100a10 was significantly up-regulated and IL34, C3, and p-STAT3 expression was markedly down-regulated. However, the effects were overturned by IL34 overexpression. The expression of IL34, C3, S100a10, and p-STAT3 were opposites when treated with miR-31-5p^{KD}-Exos. When IL34 was downregulated, the expression levels of those was reversed. Meanwhile, the expression of STAT3 has no change. These results suggested that miR-31-5p of NSCs-derived exosomes regulated IL34 and thereby modulating the STAT3 pathway.

Discussion

Traumatic spinal cord injury are the most common and severe neurological injuries, leading to motor, sensory and autonomic dysfunction. Therefore, it is a priority to explore interventions for SCI[18]. As the nanoscale of exosomes, it was able to cross the blood brain barrier (BBB). miRNA can be selectively packaged and transferred between cells in exosomes and is crucial to various pathophysiological processes[19]. Activated microglia induce the activation of astrocytes. A1 astrocytes can lead to neurotoxins release, leading to apoptosis of neurons. A2 astrocyte encourage upregulation of neurotrophic factors and the secretion of proteins promoting synaptogenesis[3].

As previously described, the therapeutic effects of NSCs could account for paracrine mechanisms. NSCs could communicate with the microenvironment via exosomes[20, 21]. Our preliminary SCI model studies demonstrated that exosomes could promote motor function recovery and axonal growth. Astrocytes included neurotoxic A1 phenotype and neuroprotective A2 phenotype. Characteristic markers for A2 phenotype include S100 calcium-binding protein A10 (S100a10), pentraxin 3 (Ptx3), and sphingosine-1-phosphate receptor 3 (S1pr3). for A1 phenotype, complement component 3 (C3), serpin family G member 1 (Serping1), and histocompatibility 2, D region locus 1 (H2d1) are major markers[3]. Our in vivo study has shown the administration of exosomes promoting astrocytes A2 polarization. We also demonstrated it in vitro. Based on these results, NSC-derived exosomes could be regarded as a bioagent to improve motor function recovery by shifting astrocytes A1/A2 phenotype following SCI.

Exosomes carry a cargo of miRNAs, mRNAs, and proteins. However, the composition of NSC-derived exosomes is different from exosomes derived from other stem cells. miRNAs and proteins contained in NSC-derived exosomes relevant for neural regeneration, neuroprotection, and neural plasticity[22]. Therefore, we investigated the mechanism that NSC-derived exosomes promoted functional recovery and shifting astrocytes A1/A2 phenotype. Present studies demonstrate that CNS derived exosomes deliver miRNAs on target cells to exert their biological functions[23, 24]. miRNA microarray reported that miR-31-5p was highly expressed in NSC-derived exosomes treated astrocytes. Moreover, miR-31-5p can be transferred efficiently by exosomes to the target astrocytes. previous studies demonstrated that miR-31-5p could partially promote astrocyte differentiation of NPCs[25]. However, The effect of NSC-derived exosomes that transmit miR-31-5p and mediate the A1/A2 phenotypic of astrocytes after SCI has not been reported yet. Our present study show that knockdown of exosomal miR-31-5p inhibited the therapeutic exosomal effect on SCI. Overexpression of exosomal miR-31-5p enhanced therapeutic exosomal effect. In this work, we determine that exosomes enriched with miR-31-5p could shift astrocytes A1/A2 phenotype and promote motor function recovery following SCI. As biological vectors, NSCs-derived exosomes could deliver biologically functional miR-31-5p into recipient astrocytes.

In order to explore the mechanism of miR-31-5p in NSCs-derived exosomes, we used bioinformatics tools to investigated the potential target gene. Therefore, IL34 was chosen as target gene for further study by RNA-ChIP and Luciferase reporter. When overexpressing miR-31-5p or upregulating knockdown miR-31-5p in astrocytes, the protein level of IL34 was downregulated. It suggested that IL34 was the potential target gene of miR-31-5p. It has been proved that mice with a deficiency in IL-34 production were protected from synapse loss during virus-induced memory impairment[26]. To further evaluate IL-34 was the target gene

of miR-31-5p, a series of experiment were performed. Our demonstrated that knockdown of IL-34 in astrocytes reversed the detrimental effects caused by inhibiting exosomal miR-31-5p expression. Overexpression of IL-34 inhibits the therapeutic effect of overexpression of miR-31-5p in exosomes. Altogether the result mentioned above, it showed that miR-31-5p in NSCs-derived exosomes could shift astrocytes A1/A2 phenotype by suppressing the IL-34 signaling pathway in the process.

Experimental study have provided that there is crosstalk between the IL-34 and STAT3 signaling pathways[16, 17]. Suppressing IL-34 could inhibit the STAT3 signaling pathway. Evidence has shown that Inhibition of STAT3 could switch A1 to A2 astrocytes phenotypic in an Alzheimer's disease model[27]. STAT3 was also involved in the activation of A1 astrocytes, and p-STAT3 was upregulated in activated A1 astrocytes in SCI[15]. To examine the signaling pathways after treated with exosomes, we detect the protein levels by western blotting. The STAT3 pathway was inhibited when administrating NSCs-derived exosomes and that were more noticeable when administration of miR-31-5p^{OE}-Exos. However, the effects of inhibiting the STAT3 pathway were partially overturned following the treatment of miR-31-5p^{KD}-Exos. These results demonstrated that miR-31-5p of NSC-derived exosomes shifting astrocytes A1/A2 phenotype by inhibiting the STAT3 signaling cascades.

Our present experiment results founded exosomal miR-31-5p derived from NSCs in shifting astrocytes A1/A2 phenotype. However, other genes cannot be ruled out which may exhibit therapeutic effects alone or combination with exosomes.

Conclusions

Our research showed that NSCs-derived exosomes promote the recovery of mouse motor function by delivering miR-31-5p. The exosomal miR-31-5p modulates the IL34/STAT3 signaling pathway to shift astrocytes from A1 to A2 phenotype. The combination of miRNAs and NSCs-derived exosomes could provide a minimally invasive treatment for SCI.

Declarations

Ethics approval and consent to participate

This study was approved by the ethics committee of Nanjing Medical University. Written informed consent was obtained from all participants included in the study. All procedures performed in studies involving animals were conducted in accordance with the animal care and guidelines of The First Affiliated Hospital of Nanjing Medical University (No. IACUC-2103040)

Consent for publication

Not applicable.

Availability of data and materials

All relevant data and materials are available from the authors upon reasonable request.

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Authors' contributions

PYT, FJ and QQY designed and supervised this study. DDJ, YJL, XSW and CYH con ducted the majority of the experiments and completed the manuscript. QFZ analyzed the data. CZJ participated the experiments and the manuscript writing. DDJ and YJL produced the spinal cord injury model. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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Figures

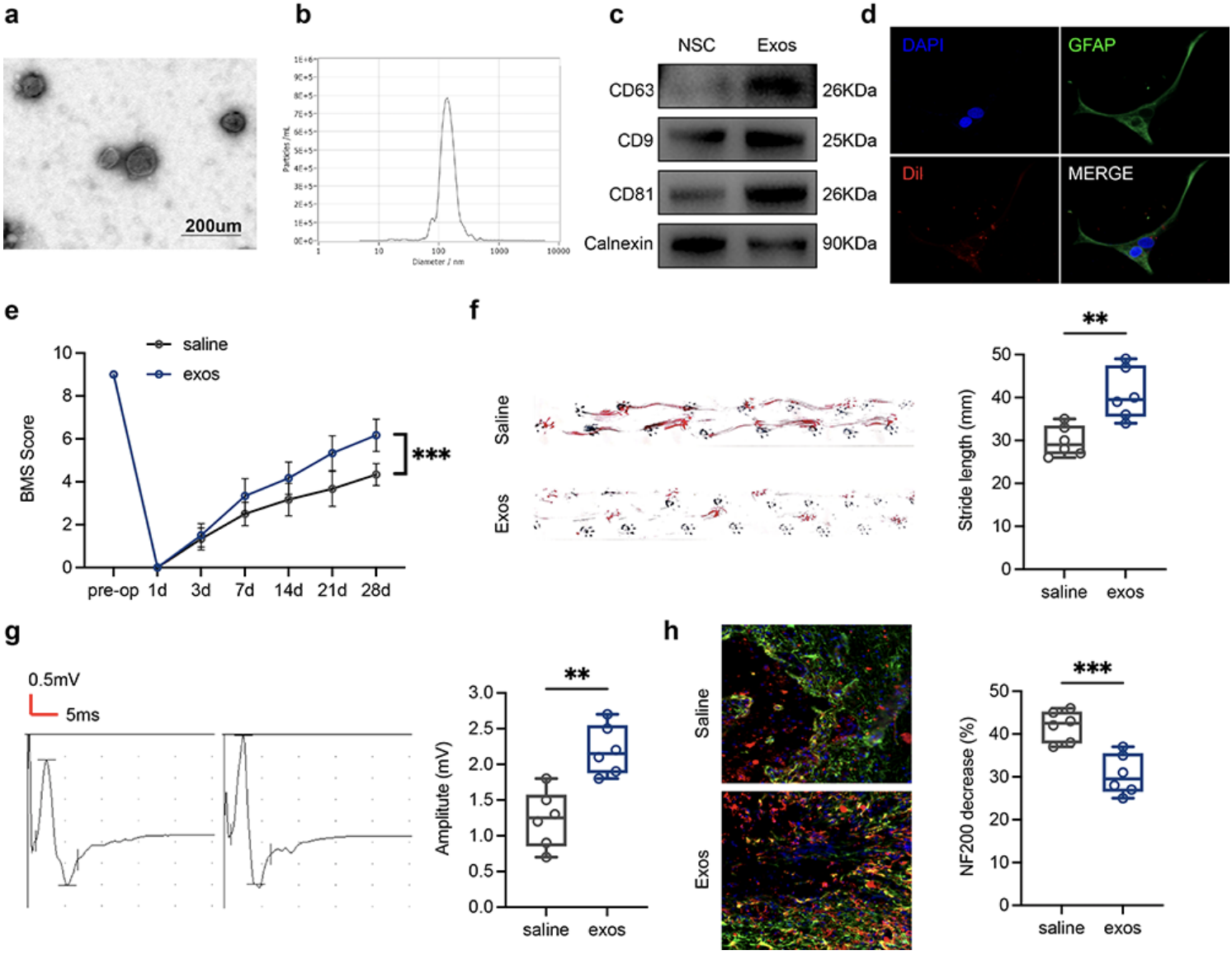

Figure 1

NSCs-derived exosomes promoted functional behavioral recovery after SCI.

a Morphology of exosomes under TEM (50–150 nm). b NTA analysis exhibit exosomes size. c Western blot analysis of exosome surface markers. d Uptake of the red fluorescence dye DiI-labeled Exos into astrocytes. e BMS was used to functionally grade in the Saline and Exos groups at 28 days after SCI for mice. f The footprints quantification of mice walking 28 days after SCI. red: hindpaw print, Blue: frontpaw print. g Electrophysiological assessment in two groups at 28 days after SCI. h Immunostaining of NF200 and GFAP in the injured areas of spinal cord at 28 days after SCI. *P < 0.05

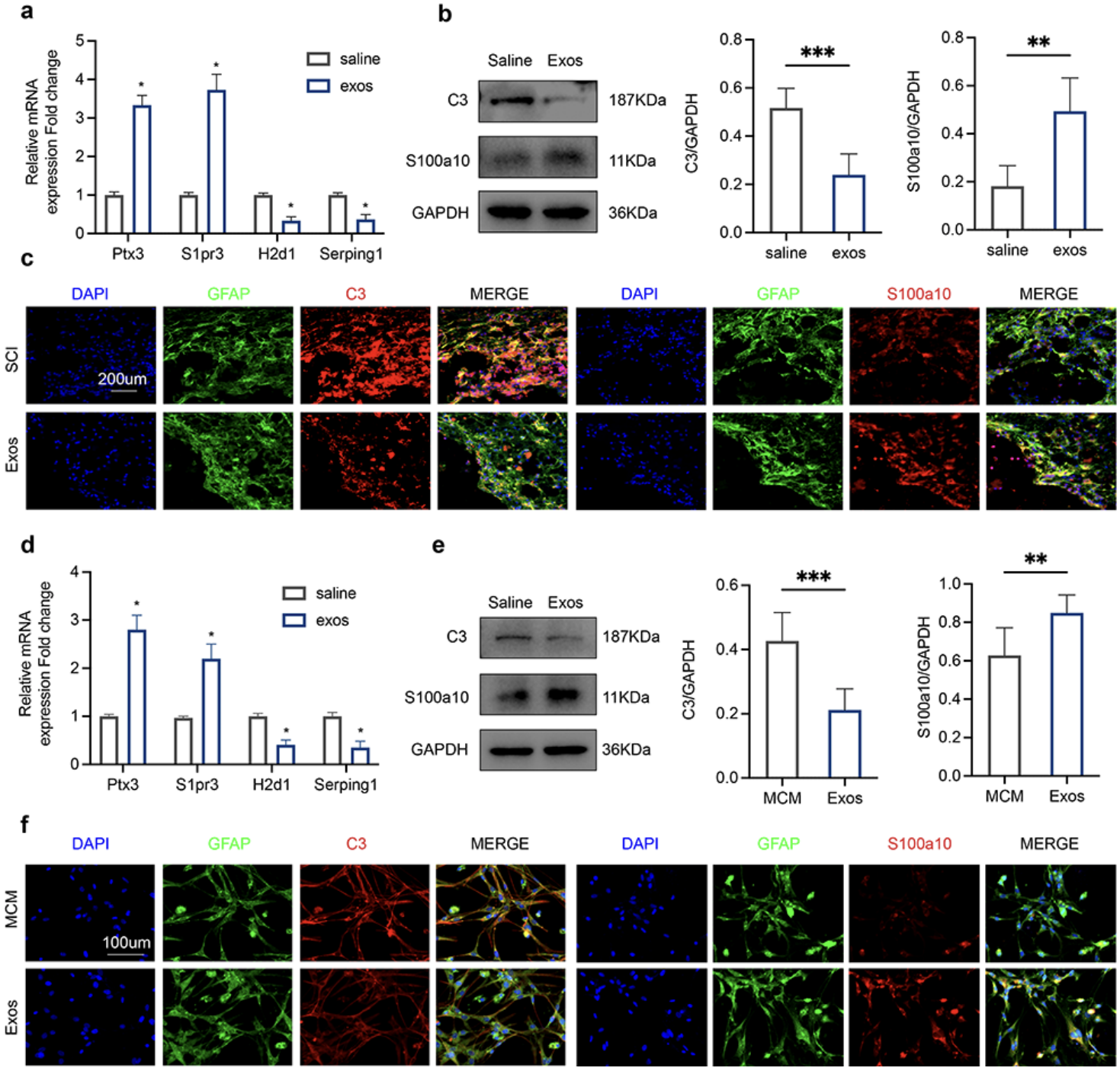

Figure 2

Administration of Exos following SCI promoted astrocytes polarization from A1 to A2 phenotype.

a The mRNA expression levels of A1- and A2-related genes were detected by qRT-PCR (n = 6/group). b The protein levels of A1- and A2-related genes were detected by western blot analysis (n = 6/group). c Representative immunostaining image of GFAP (green) and C3/S100a10 (red) in the injured spinal cord lesion areas at day 28 post-injury (n = 6/group). d The mRNA expression levels of A1- and A2- related genes were detected by qRT-PCR in astrocytes in the PBS and Exos groups. e The protein expression

levels of A1- and A2-related genes were detected by western blot in astrocytes in two groups. f Representative immunostaining image of GFAP (green) and C3/S100a10 (red) in astrocytes. *P < 0.05

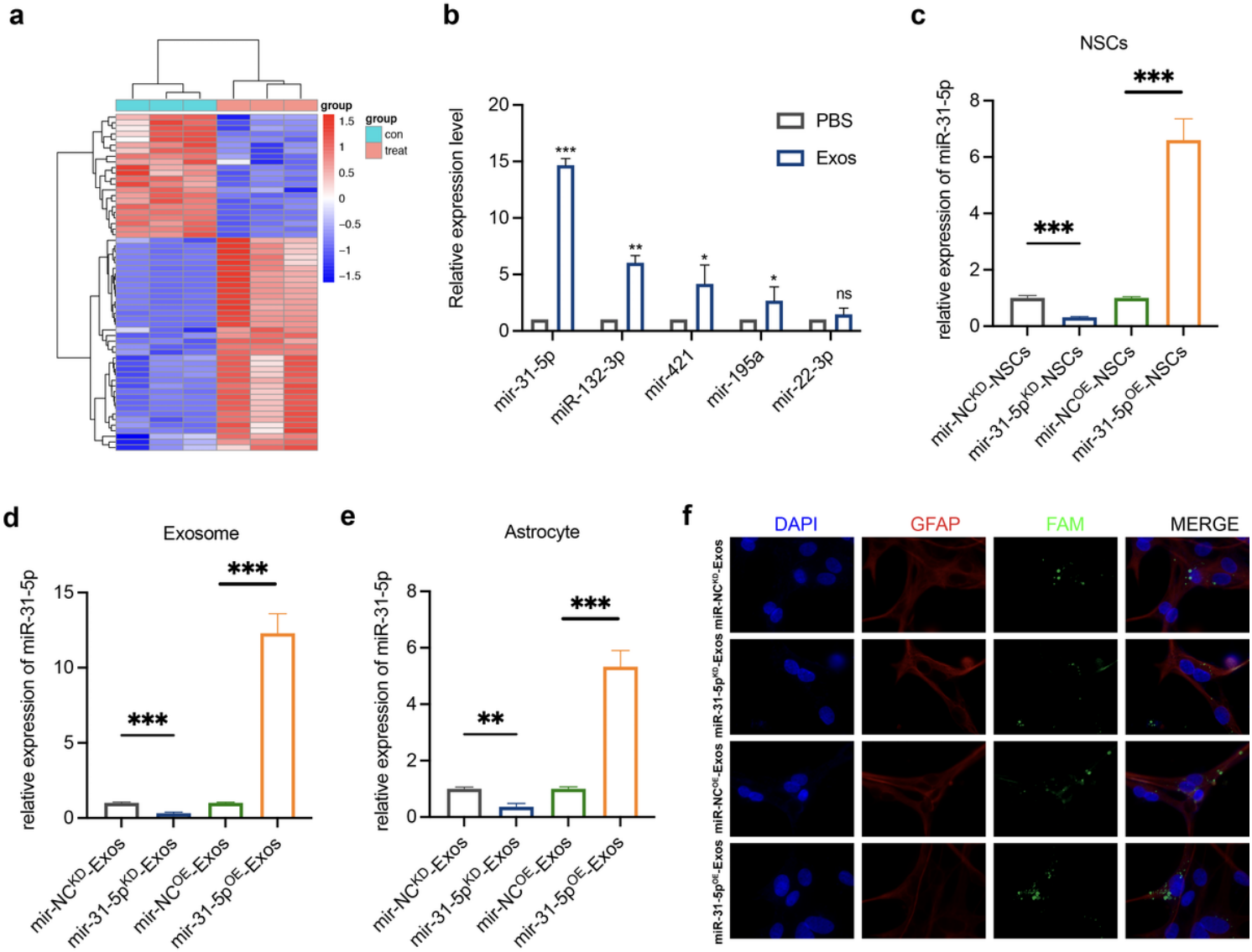

Figure 3

miR-31-5p transferred to astrocytes by exosomes and upregulated.

a miRNAs in Heat map with a ≥ 1.5-fold difference between astrocytes treated with exosomes and PBS. b qRT-PCR was used to compare the top five elevated miRNAs between astrocytes treated with exosomes and PBS. c mir-31-5p knockdown and overexpression in NSCs and the efficiency was confirmed using qRT-PCR. d The relative expression level of mir-31-5p in exosomes derived from NSCs transfected with mir-31-5p^{OE}, miR-NC^{OE}, mir-31-5p^{KD} and miR-NC^{KD}. e Expression level of mir-31-5p in astrocytes after administering miR-NC^{KD}-Exos, mir-31-5p^{KD}-Exos, miR-NC^{OE}-Exos and mir-31-5p^{OE}-Exos. f Representative images of FAM-labeled exosomal mir-31-5p internalized by astrocytes after administration of miR-NC^{KD}-Exos, mir-31-5p^{KD}-Exos, miR-NC^{OE}-Exos and mir-31-5p^{OE}-Exos. *P < 0.05, ns indicates no significance

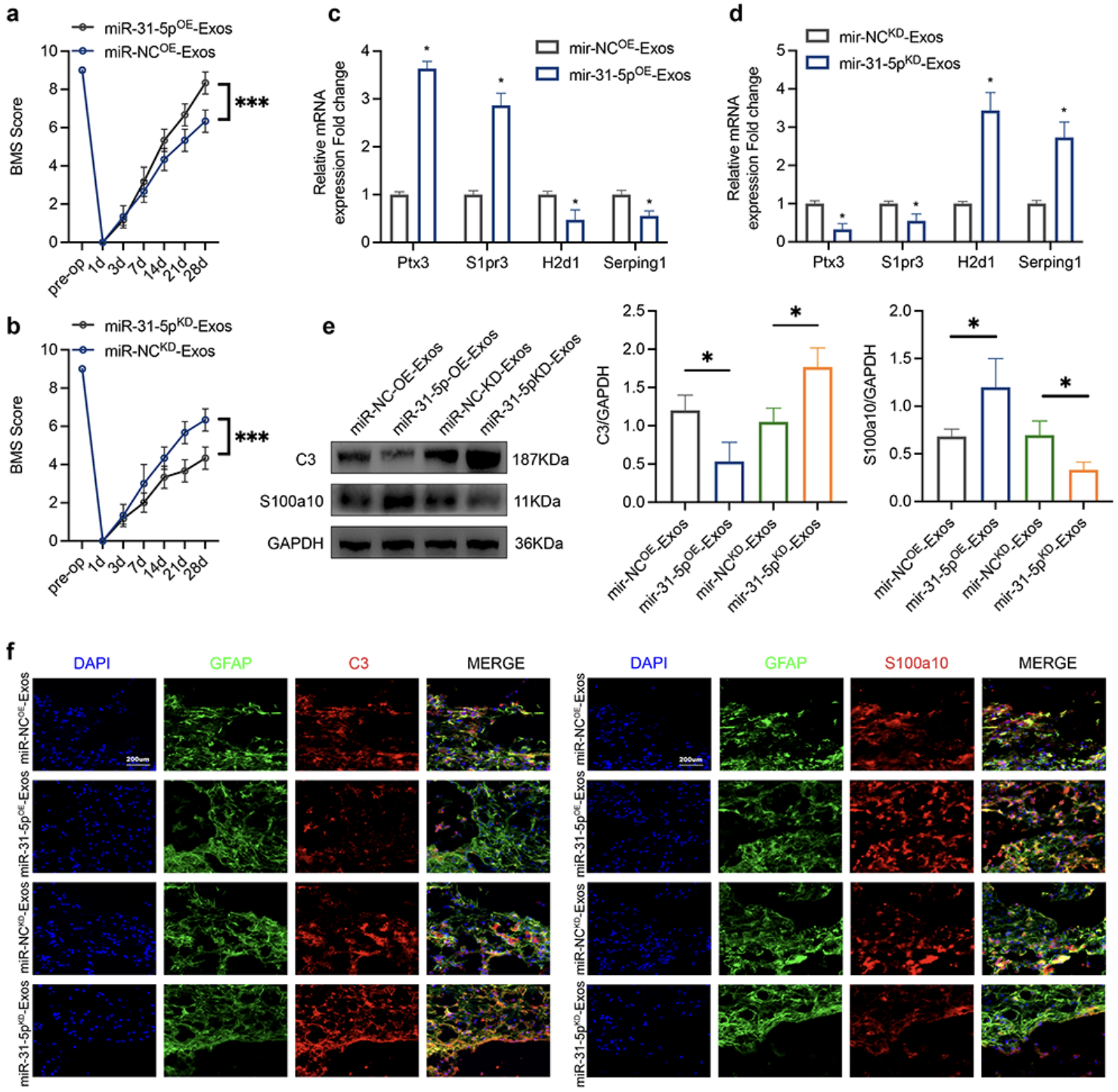

Figure 4

Exos shifted astrocytes polarization from A1 to A2 phenotype by delivering mir-31-5p in vivo.

a and b BMS was used to functionally grade at 28 days after SCI for mice. c and d The mRNA expression levels of A1- and A2-related genes were detected by qRT-PCR (n = 6/group). e The protein levels of A1- and A2-related genes were detected by western blot analysis (n = 6/group). f Representative immunostaining image of GFAP (green) and C3/S100a10 (red) in the injured spinal cord lesion areas at day 28 post-injury (n = 6/group). *P < 0.05

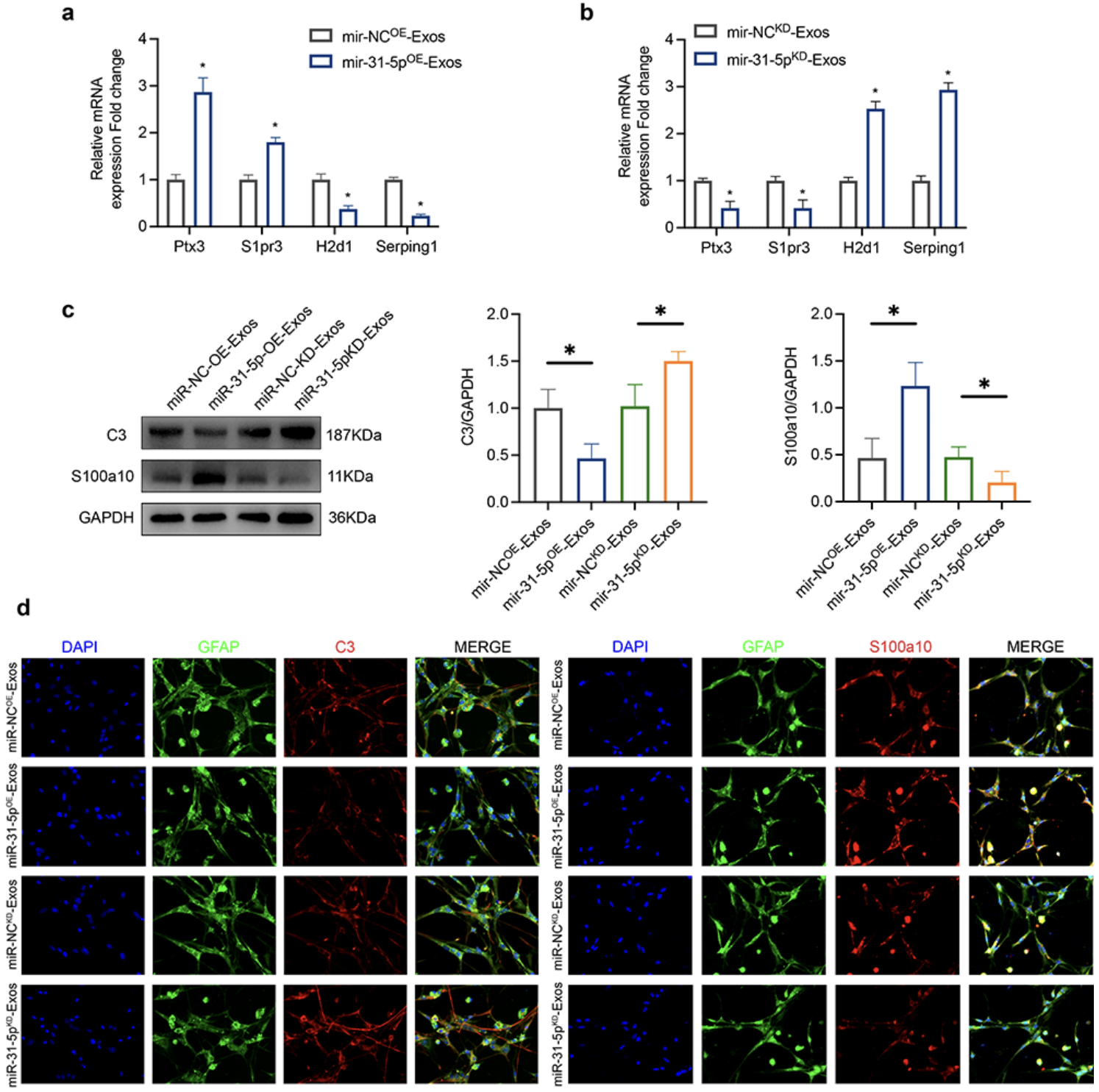

Figure 5

Exos shifted polarization from A1 to A2 in astrocytes by shuttling mir-31-5p in vitro.

a and b The mRNA expression levels of the A1- and A2-related genes were detected by qRT-PCR in astrocytes in the different groups. c The protein expression levels of A1- and A2-related genes were detected by western blot in astrocytes in the different groups. d Representative immunostaining image of GFAP (green) and C3/S100a10 (red) in astrocytes. *P < 0.05

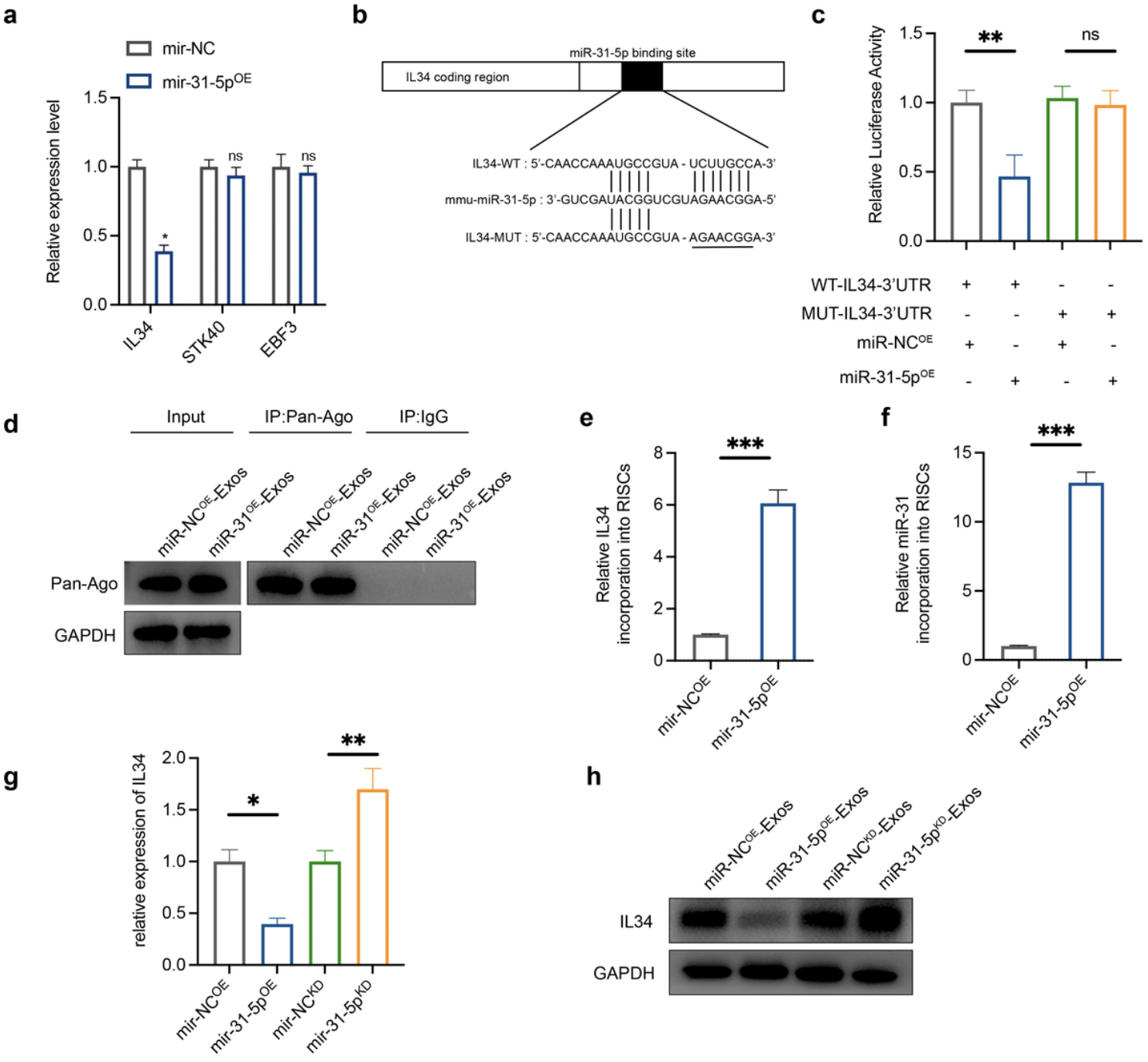

Figure 6

Exosomal mir-31-5p regulates IL34 by directly targeting the 3'-UTR.

a The mRNA expression levels of three predicted target genes in astrocytes after administration of miR-NC-Exos and mir-31-5p^{OE}-Exos. b and c Luciferase reporter assay confirmed that IL34 was the target gene of mir-31-5p. d-f Immunoprecipitation of the Ago2/RISC (RNA-induced silencing complex) using the Pan-Ago2 antibody in astrocytes overexpressing miR-NC or mir-31-5p. IgG was used as a negative control and GAPDH was used as an internal control. g The mRNA expression level of IL34 in astrocytes after mir-31-5p knockdown and overexpression by qRT-PCR. h Western blot analysis of IL34 in astrocytes after mir-31-5p overexpression and knockdown. *P < 0.05

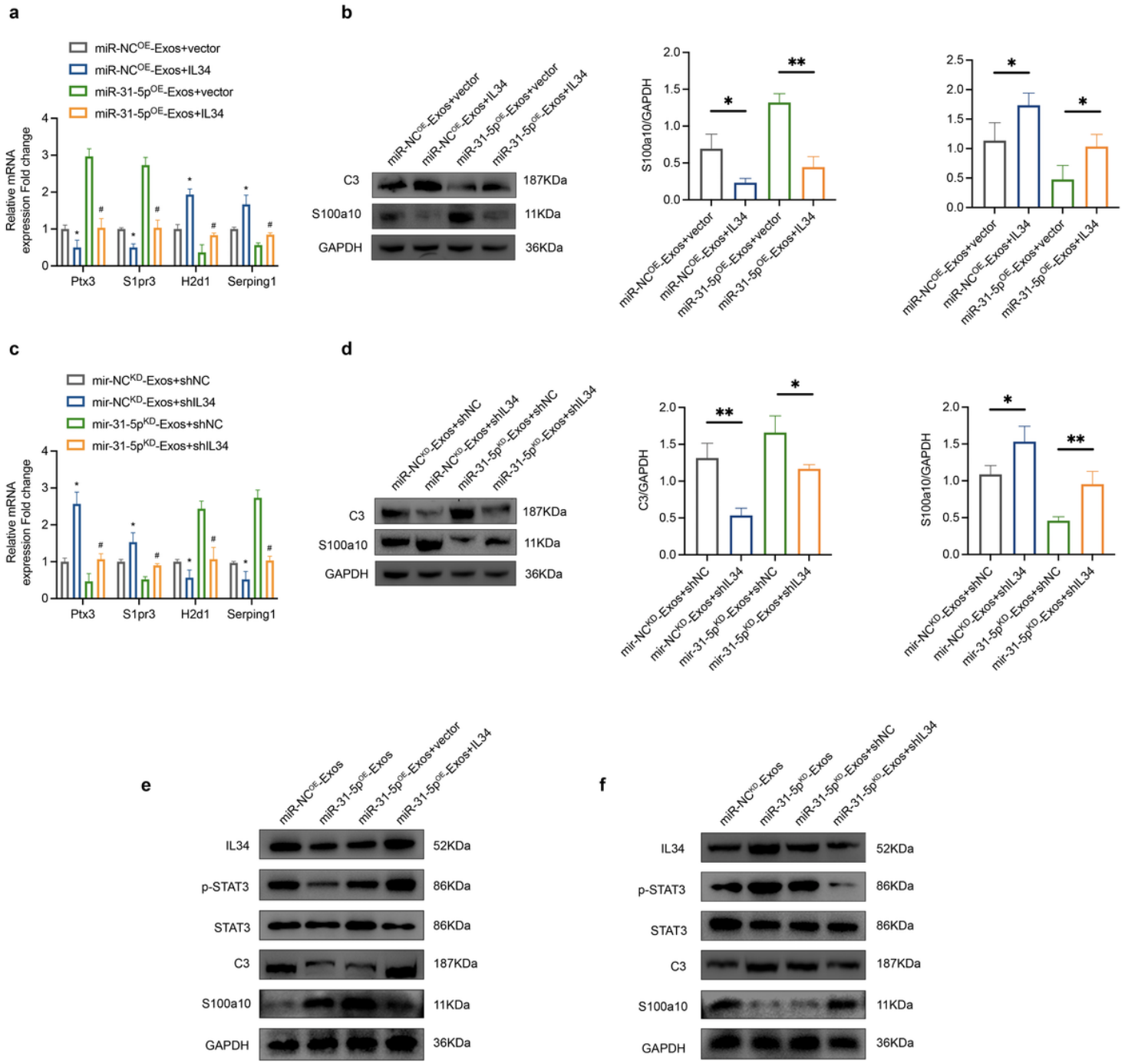

Figure 7

Exosomal mir-31-5p regulates astrocyte A1/A2 polarization through IL34/STAT3 signaling cascades.

A series of gain- and loss-of-function experiments including qRT-PCR (a and c) and western blot (b and d) were carried out to verify the functional role of IL34 on astrocyte polarization in astrocytes. astrocyte A1/A2 polarization was detected by qRT-PCR (a and c) and western blot analysis (b and d). e Representative images of western blots for IL34 downstream STAT3 signaling cascades in astrocytes when administrating miR-NC^{OE}-Exos, mir-31-5p^{OE}-Exos, mir-31-5p^{OE}-Exos + vector and mir-31-5p^{OE}-Exos + IL34. f Representative images of western blots for IL34 and downstream STAT3 signaling cascades in

astrocytes when administrating miR-NC^{KD}-Exos, mir-31-5p^{KD}-Exos, mir-31-5p^{KD}-Exos+shNC and mir-31-5p^{KD}-Exos+shIL34.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS1.pdf
- FigureS2.pdf
- Table1.xlsx