Mesenchymal Stem Cell-Derived Extracellular Vesicles
Alleviate M1 Microglial Activation in Brain Injury of Mice
with Subarachnoid Hemorrhage via microRNA-140-5p Delivery

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Abstract

Background: It is documented that mesenchymal stem cells (MSCs) secrete extracellular vesicles (EVs) to modulate subarachnoid hemorrhage (SAH) development. miR-140-5p expression has been detected in MSC-derived EVs, while it remains enigmatic about the mechanism of MSC-derived EVs containing miR-140-5p in SAH. We aim to fill this void by establishing SAH mouse models and extracting MSCs and MSC-EVs.

Methods: After ALK5 was silenced in SAH mice, neurological function was evaluated, neuron apoptosis was detected by NeuN/TUNEL staining, and expression of serum inflammatory factors (IL-6, IL-1β, and TNF-α) was determined by ELISA. Effect of ALK5 on NOX2 expression was assessed by western blot analysis. Targeting relationship between miR-140-5p and ALK5 was evaluated by dual luciferase assay. Following extraction of MSCs and MSC-EVs, EVs and miR-140-5p were labeled by PKH67 and Cy3 respectively to identify the transferring of miR-140-5p by MSC-EVs. SAH mice were treated with EVs from miR-140-5p mimic/inhibitor-transfected MSCs to detect effects of MSC-EV-miR-140-5p on brain injury and microglial polarization.

Results: ALK5 silencing increased neurological score, and reduced neuron apoptosis and neuroinflammation in SAH mice. ALK5 silencing inhibited M1 microglia activation by inactivating NOX2. ALK5 was a target gene of miR-140-5p. MSC-derived EVs contained miR-140-5p and transferred miR-140-5p into microglia. MSC-EV-delivered miR-140-3p reduced ALK5 expression to contribute to repression of brain injury and M1 microglia activation in SAH mice.
Conclusions: MSC-derived EVs transferred miR-140-5p into microglia to downregulate ALK5 and NOX2, thus inhibiting M1 microglia activation in SAH mice.

Keywords: Subarachnoid hemorrhage; M1 microglia activation; Brain injury; Mesenchymal stem cells; Extracellular vesicles; microRNA-140-5p; ALK5; NOX2
Introduction

As one of the most prevalent and dangerous forms of cerebrovascular disease, subarachnoid hemorrhage (SAH) mainly results from sudden rupture of an intracranial aneurysm (Pang et al., 2019). Although it is less common than ischemic stroke, SAH has a higher mortality rate, estimated to be in the range of 32% to 67% or nearly 40% in more recent studies (Geraghty and Testai, 2017). The brain injury that occurs directly after an SAH event is multimodal, including early brain injury and delayed brain injury (Schneider et al., 2018). Moreover, extravasation of blood after aneurysm rupture contributes to a strong inflammatory response featured by the activation of microglia (Geraghty et al., 2019). Meanwhile, prior research has shown that microglia activation-induced neuroinflammation reaction plays a crucial part in early brain injury after SAH (Xu et al., 2019). Hence, this process might present a promising therapeutic strategy for SAH, giving new insights into the molecular mechanism underlying microglia activation in SAH.

As reported previously, mesenchymal stem cells (MSCs) involve in activated microglia-caused neuroinflammation post-SAH (Zhang et al., 2020). Importantly, MSCs release extracellular vesicles (EVs) to decrease microglia-modulated neuroinflammation following perinatal brain injury (Thomi et al., 2019). Interestingly, MSC-derived EVs could attenuate early brain injury after SAH (Xiong et al., 2020). As is known to all, MSC-EVs are able to deliver lipids, proteins, enzymes, microRNAs (miRs), and mRNAs to be endowed with anti-inflammatory effects (Elia et al., 2019). A study illustrated overexpression of miR-140-5p in EVs derived from MSCs (Tao et al., 2017). Notably, miR-140-5p correlates to cell proliferation and differentiation, and is involved in cancer initiation and development (Lu et al., 2019; Wu et al., 2019). Furthermore,
Data obtained by Wang et al. unraveled the inhibitory effect of miR-140-5p on neuroinflammation and brain injury in rats with intracerebral hemorrhage (Wang et al., 2019).

miRs play pivotal roles in post-transcriptional silencing of target genes by binding to 3'-untranslated region (3'−UTR) of mRNAs (Hammond, 2015). The putative target genes of miRs can be predicted by in-silico algorithms from websites, including TargetScan website (Lu and Rothenberg, 2018). By utilizing this method, we found activin-like kinase 5 (ALK5) as a potential target of miR-140-5p. ALK5, also named as TGF-β type I receptor (TβRI), is a member of the family of serine/threonine protein kinases (Ungefroren et al., 2018). Moreover, a prior study uncovered that treatment with ALK5 inhibitor Sb505124 could alleviate cerebral ischemia/reperfusion injury by downregulating NADPH oxidase 2 (NOX2) (Lou et al., 2018). More importantly, NOX2 was overexpressed in activated M1 microglia of mice with experimental SAH (Pang et al., 2018). Considering these studies, a hypothesis was proposed that MSC-derived EV containing miR-140-5p reduced M1 microglia activation following SAH by downregulating ALK5 and NOX2. Hence, we undertook the present study to test this hypothesis in a murine SAH model and mechanisms related to ALK5 and NOX2.

Materials and methods

Compliance with ethical standards

Animal experiments were conducted under the approval of Animal Ethics Committee of the Affiliated People’s Hospital of Jiangsu University and in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of
Health. Efforts were made to minimize pain, suffering and discomfort to experimental animals.

**SAH model establishment in mice**

Thirty-six male C57BL/6J mice (aged 8-10 weeks, weighing 18-22 g, all from Animal Research Center of Jiangsu University, Zhenjiang, China) or NOX2 knockout (NOX2−/−) mice (Cyagen Biosciences Inc., Suzhou, China) were placed in a supine position after induction of anesthesia by intraperitoneal injection of sodium pentobarbital (50 mg/kg). The right external carotid artery (ECA) and internal carotid artery (ICA) were exposed after incision of the neck at the midline. A 5-0 monofilament nylon wire (Ethion, Somerville, New Jersey, USA) was implanted to penetrate bifurcation of anterior cerebral artery (ACA) and middle cerebral artery (MCA) through ECA and ICA. In sham-operated mice, the same procedure was performed without penetration of blood vessels. All operations were performed from 9:00 a.m. to 12:00 a.m.

**Extraction and identification of MSCs**

The femur and tibia of both hindlimbs of adult male C57BL/6J mice (weighing 18-22 g) were harvested. Then, the bone marrow plugs were then rinsed from the medullary cavity using a 10-ml syringe encompassing low-glucose DMEM (Sigma-Aldrich, MO, USA). Mononuclear cells were obtained by centrifuging bone marrow cell suspension at 400 ×g for 20 min, and incubated in culture flasks at 1 × 10^6 cells/25 cm^2 at 37°C with 5% CO₂. Upon 90% confluence, adherent cells were trypsinized and passaged. The subsequent experiments were conducted on bone marrow-derived MSCs which were passaged four times. Flow cytometry was performed to detect typical positive markers (CD13 [clone number: R3-242], CD90 [clone number: 53-2.1], and CD105 [clone number: R12-101])
number: MJ7/18]) and a negative marker (CD45 [clone number: 30-F11]) for MSCs [BD Biosciences Pharmingen (San Jose, CA, USA), the used concentration of 1 μg/mL]. MSCs of passage 3-7 were cultured in osteogenic, adipogenic, or chondrogenic differentiation media [all from Cyagen Biosciences Inc. (Guangdong, China)]. Osteogenic, adipogenic, and chondrogenic differentiation were identified by alkaline phosphatase (ALP), oil red O, and Alcin blue staining.

**Isolation and characteristics of EVs from MSCs**

In a 10-cm culture dish, MSCs were cultured in EV-free serum medium (14-h centrifugation at 120000 ×g and 4°C) for 72 h. When cell confluence reached 90-100%, the culture medium was centrifuged for 10 min at 3000 ×g and 4°C to remove cells and then at 10000 ×g and 4°C for 30 min to remove large EVs. The collected supernatant was filtered through a 0.22 µm filter (Millipore, Burlington, MA, USA) to remove microvesicles and contaminating apoptotic bodies. Then the supernatant was ultracentrifuged with Optima L-80XP (Beckman Coulter, Brea, CA, USA) at 100000 ×g and 4°C for 2 h. Pellets were resuspended in ice phosphate buffer saline (PBS) and centrifuged at 100000 ×g and 4°C for 2 h, followed by resuspension of EVs in 200 µL ice PBS.

Dynamic light scattering (DLS) of Nanosizer™ instrument (Malvern Instruments, Malvern, UK) was utilized to measure size distribution of MSC-derived EVs. Diluted EVs were injected into Nanosight NS300 instrument. Afterwards, particles were automatically tracked and size was identified in accordance to Brownian motion and diffusion coefficient. The morphology of EVs was observed using a Hitachi H-7650 transmission electron microscope (TEM, Hitachi, Tokyo, Japan). Then 10 µL EV particles
were positioned on formvar carbon-coated 200-mesh copper electron microscopy grids, followed by 5-min incubation. Then, EV particles underwent 1-min standard 1% uranyl acetate staining. Observation was conducted under a TEM. The characteristics of EVs were identified by detecting EV-specific surface marker expressions, including CD63 (1: 2000, ab216130, rabbit, Abcam, Cambridge, UK), tumor susceptibility gene 101 (TSG101; 1: 10000, ab125011, rabbit, Abcam), CD9 (1: 2000, A19027, rabbit, ABclonal, Wuhan, China), and cis-Golgi matrix protein of 130 kD (GM130; 1: 20000, A11408, rabbit, ABclonal) by Western blot analysis.

**Cell culture**

The semi-adherent mouse cell line BV2 (ATL03001, the National Infrastructure of Cell Line Resource) was cultured with DMEM H-21 4.5 g/Liter Glucose (DMEM-H) containing 10% FBS (Sigma-Aldrich). Mechanical vibrations and PBS rinsing were performed to detach BV2 cells from the culture plates.

**Cell transfection and transduction**

Transfection was carried out on the 60% confluent MSCs and BV2 by Lipofectamine 2000 reagent (Invitrogen, CA, USA). Synthetic miR-140-5p mimic, mimic-negative control (NC), miR-140-5p inhibitor, and inhibitor-NC were from RiboBio (Guangdong, China), whereas lentiviral packaging overexpression plasmid of ALK5, control vector plasmid and lentiviral packaging short hairpin (sh) ALK5 and control short hairpin RNA (shRNA) were from Genecopoeia (Rockville, MD, USA). Lentiviral infection was utilized to construct ALK5-knockdown BV2 cells. The sequence of shALK5 was CAGAATACAGCACCACAAAATCCTC.
EV internalization

Initially, $5 \times 10^5$ cells were suspended in 500 μL EV-free medium and incubated with PKH67-labeled EVs (2 μg) at 37°C for a specified time, followed by confocal microscopic observation. Cells were frozen, and fluorescence of living cells was observed with Leica TCS-STED confocal microscope (Leica Microsystems, Heidelberg, Germany).

Treatment of SAH mice

SAH mice underwent intracerebroventricular injection of shRNA lentivirus or overexpression lentivirus: mice were placed in a stereotaxic apparatus and anesthetized with isoflurane (4% for induction, 2.5% for maintenance), shRNA lentivirus and overexpression lentivirus (the titer of $1 \times 10^8$ TU/mL) were injected into the right lateral ventricle using: 1.5 mm posterior, 1.0 mm lateral, and 3.2 mm below the horizontal plane of the bregma. The same volume of control lentivirus was used as a NC. Then 200 μL lentivirus was injected into right ventricle through a syringe pump at 1 μL/min at 48 h before SAH induction. After injection, the needle was kept in place for another 5 min, and retracted slowly. The femoral vein was injected with 200 μL EVs at 1 h after SAH modeling.

SAH grading

An investigator blinded to experimental groups evaluated SAH grading score at 24 h after SAH. Briefly, the basal cistern was divided into six segments, and each segment was scored into grade ranging from 0 to 3, where 0 indicated no obvious subarachnoid blood clot, 1 grade referred to a minor blood clot, 2 grade referred to a moderate blood clot, and 3 grade referred to a large subarachnoid blood clot with an invisible Willis...
A total score of six segments defined the SAH grade of mice (exemplars of each grade are shown in Supplementary Materials) (Sugawara et al., 2008; Xie et al., 2020).

**Neurological Score**

At 24 h after SAH modeling, a blinded investigator assessed the neurological performances of all animals using modified Garcia neurological score. The neurological function was evaluated by tests of spontaneous activity (score 0 - 3), spontaneous movement of four limbs (score 0 - 3), forepaw outstretching (score 0 - 3), climbing (score 1 - 3), body proprioception (score 1 - 3), and responses to vibrissae touch (score 1 - 3). Lower scores indicate severe neurological deficits (Pang et al., 2018).

**Neuron apoptosis detection**

At 24 h after SAH induction, mice were perfused with 0.1 M PBS and 4% paraformaldehyde. The whole brain was soaked in 4% paraformaldehyde at 4°C for 24 h, followed by dehydration in 30% sucrose solution. After being frozen, the brains were cut into 8-μm slices with a cryostat microtome (Leica CM3050S-3-1-1, Leica Microsystems Inc. Bannockburn, IL, USA). The overnight culture of brain slices was performed at 4°C with a primary neuronal nuclei (NeuN) antibody (ab177487, 1: 500, Abcam). Afterwards, the slices were reprobed with red fluorescein-conjugated secondary antibody (ab150079, Abcam). Neuron apoptosis was determined by TUNEL staining (11772457001, Roche Inc., Basel, Switzerland). A fluorescence microscope (Olympus, Tokyo, Japan) was utilized to observe the slices. The apoptotic degree of neurons was evaluated with percentage of TUNEL positive in 6 sections as apoptotic index.
ELISA

The protein levels of tumor necrosis factor alpha (TNF-α, CSB-E04741M), Interleukin-beta (IL-1β, CSB-E08054M), and IL-6 (CSB-E04639M) in serum were detected by ELISA kits (CUSABIO, Wuhan, China). Frozen right cerebral hemisphere was mechanically homogenized and centrifuged at 12000 rpm and 4°C for 15 min. Inflammatory cytokine levels were quantified using a mouse-specific ELISA kit.

Microglia phenotype detection

The 8-μm frozen sections of cerebral cortex and cells were fixed, permeated with 0.3% Triton X-100, and blocked with 1% bovine serum albumin (BSA). Then, sections/cells were incubated with primary antibodies to CD16 (1: 200, A13980, ABclonal), CD206 (1: 100, ab195191, Abcam), and Iba 1 (1: 100, A12391, ABclonal) overnight at 4°C, and incubated with corresponding fluorescent secondary antibodies (AS011 and AS007, ABclonal). Finally, images were captured under a confocal laser scanning microscope (FluoView FV10i, Olympus; 40 ×) in three randomly selected fields from each section (five sections were randomly selected from each mouse). ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) was adopted for cell counting and fluorescence intensity analysis.

RT-qPCR

TRIzol reagent (Invitrogen) was adopted for total RNA extraction from cultured cells. For mRNA detection, cDNA was synthesized from 1 μg total RNA using a Revert Aid first-strand cDNA synthesis kit (Fermentas Life Sciences, Burlington, Canada). RT-qPCR analysis was performed on SYBR Premix ExTaq™ II in an ABI PRISM® 7900HT System.
(Takara, Tokyo, Japan). The relative mRNA expression was determined by $2^{-\Delta \Delta CT}$ normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For miR analysis, EV miRs were isolated by SeraMir EVssome RNA Purification Kit (System Biosciences, Mountain View, CA, USA). The cDNA of the miR was synthesized using TaqMan microRNA assay kit (Applied Biosystems, Foster City, CA, USA). RT-qPCR reaction was conducted using FastStart Universal SYBR Green Master Mix (Roche Inc.) with the miRNA-specific forward primer (Sangon Biotech, Shanghai, China) and universal reverse primers provided by TaqMan microRNA assay kit. Results were normalized using Cel-miR-39-3p small nuclear RNA. The PCR primers used are shown in Supplementary Table 1.

**Western blot analysis**

Total protein was extracted from cerebral cortex or BV2 cells using radioimmunoprecipitation assay lysis buffer (P0013C, Beyotime, Shanghai, China) with phosphatase inhibitor or protease inhibitor, with the concentration estimated by bicinchoninic acid protein quantification kit (AR0146, BOSTER, Hubei, China). Afterwards, protein was subjected to separation by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electroblotted into a polyvinylidene fluoride membrane which was sealed by 5% BSA for 2 h. The diluted primary rabbit antibodies (ABclonal) to ALK5 (1: 2000, A0708) and NOX2 (1: 2000, ab180642) were supplemented into the membrane for overnight incubation at 4°C. The following day, the membrane was incubated with horseradish peroxidase-tagged secondary antibodies (ABclonal) to goat anti-rabbit Immunoglobulin G (IgG) (1: 10000, AS014) or goat anti-mouse IgG (1: 10000, AS003) for 1 h. The protein bands were visualized using enhanced chemiluminescence reagent (Thermo Fisher Scientific, Waltham, MA, USA),
photographed using ChemiDoc XRS Plus luminescent image analyzer (Bio-Rad, Hercules, CA, USA), and quantified by Image J analysis software, with GAPDH (1: 50000, AC033, mouse, ABclonal) as the loading control. The antibodies are shown in Supplementary Table 2.

**Dual luciferase reporter assay**

The 293T cells (from Cell Bank of Shanghai Institute of Cells, Chinese Academy of Sciences, Shanghai, China) were cultured in glucose DMEM. The 80 - 90% confluent cells were trypsinized and passaged, followed by conventional culture in a 5% CO₂ incubator at 37°C. The subsequent experiments were conducted on the cells at logarithmic growth phase. The upstream miR analysis of ALK5 was performed using the biological prediction website targetscan.org, and then dual luciferase reporter assay was adopted to verify whether ALK5 was a direct target of miR-140-5p. ALK5 3’-UTR containing DNA fragment of the target site of miR-140-5p was artificially synthesized, and then cloned into pGL3 vector (Promega, Madison, WI, USA) using the endonuclease site. Complementary sequence mutation sites of seed sequences were designed on ALK5 wild type (WT). The target fragments were inserted into pGL3-control vector. The correctly sequenced luciferase reporter plasmids WT and mutant type (MUT) were co-transfected with miR-140-5p mimic to 293T cells, respectively. Luciferase activity was detected on a Luminometer TD-20/20 detector (E5311, Promega) using a Dual-Luciferase Reporter Assay System kit (E1910, Promega).
Statistical analysis

All measurement data were shown as mean ± standard deviation and analyzed by SPSS 21.0 software (IBM Corp., Armonk, NY, USA), with $p < 0.05$ as a level of statistically significance. If conforming to normal distribution and homogeneity of variance, data between two groups were compared by unpaired $t$ test, while comparisons among multiple groups were performed using one-way analysis of variance.

Results

Inhibition of ALK5 alleviated brain damage in mice after SAH

It has been shown that inhibition of ALK5 can attenuate cerebral ischemia/reperfusion injury (Zhang et al., 2019), but its role in the brain injury caused by SAH is still unidentified. In order to study role of ALK5 in the brain injury caused by SAH, SAH mouse model was established, in which ALK5 was silenced. Western blot displayed higher ALK5 expression in brain tissues of SAH mice than sham-operated mice. Conversely, ALK5 expression was reduced in SAH mice treated with sh-ALK5 (Figure 1A; Supplementary Figure 1A). Compared with sham-operated mice, SAH grade in SAH mice, SAH mice treated with sh-NC and sh-ALK5 was aggravated (Figure 1B). In comparison with the sham-operated mice, all SAH mice had decreased neurological function, but sh-ALK5 overexpression appreciably enhanced neurological function of SAH mice (Figure 1C). Detection of neuron apoptosis in cerebral cortex of SAH mice also found that number of TUNEL/NeuN positive cells was reduced in SAH mice by silencing ALK5 (Figure 1D). Meanwhile, from ELISA results, inflammatory factor (IL-6, IL-1β, and TNF-α) expression was higher in SAH mice than sham-operated mice. Decrease of IL-6, IL-1β, and TNF-α expression was observed in ALK5-slienced SAH mice (Figure 1E).
Thus, inhibition of ALK5 could decrease neurological dysfunction and neuroinflammation in mice after SAH.

**ALK5 silencing reduced M1 microglia activation through NOX2 downregulation in SAH mice**

It has been documented that activation of microglia is associated with brain injury. Therefore, the effect of ALK5 on microglia activation was detected, which manifested that silencing of ALK5 strikingly reduced number of CD16-positive M1 microglia and increased number of CD206-positive M2 microglia in the cerebral cortex (Figure 2A; Supplementary Figure 2A). Prior studies have shown that NOX2 promotes M1-like microglia activation after experimental traumatic brain injury (Kumar et al., 2016), and that inhibition of ALK5 reduces NOX2 expression after cerebral ischemia/reperfusion injury (Zhang et al., 2019). Then, as depicted in Figure 2B; Supplementary Figure 1B, after ALK5 was silenced in SAH mice, NOX2 was remarkably diminished in brain tissues. To verify the effect of NOX2 on microglial polarization, we selected NOX2 knockout mice (NOX2−/−). After SAH treatment, compared with WT mice, number of CD16-positive M1 microglia in cerebral cortex of NOX2−/− mice was prominently reduced while that of CD206-positive M2 microglia was increased (Figure 2C; Supplementary Figure 1C; Supplementary Figure 2B). This indicated that the knockout of NOX2 reduced the activation of M1 microglia in SAH mice. After pretreatment of NOX2−/− mice with oe-ALK5 lentiviral vector, AKL5 expression increased in the cerebral cortex (Figure 2D; Supplementary Figure 1D), but the number of CD16-positive M1 and CD206-positive M2 microglia was not affected (Figure 2E; Supplementary Figure 2C). This result suggested that knockout of NOX2 blocked the effect of ALK5 on microglial polarization. In
conclusion, ALK5 silencing decreased NOX2 expression to repress M1 microglia activation in SAH mice.

**ALK5 is a target gene of miR-140-5p in BV2 cells**

Subsequently, we explored upstream mechanism of ALK5 in SAH. Firstly, it was predicted by TargetScan website that miR-140-5p had binding sites at ALK5 3'UTR 389-396 (Figure 3A). It was reported that miR-140-5p could attenuate intracerebral hemorrhage-induced brain injury and neuroinflammation in rats (Wang et al., 2019). Hence, we speculated that miR-140-5p might inhibit brain injury by downregulating ALK5. Reduction of luciferase activity was found in ALK5 3'UTR-WT after treatment with miR-140-5p mimic and no significant change of luciferase activity in ALK5 3'UTR-MUT (Figure 3B). In addition, after miR-140-5p mimic was transfected into mouse microglia BV2 cell, miR-140-5p expression was elevated (Figure 3C) while ALK5 and NOX2 expression was decreased (Figure 3D, E; Supplementary Figure 1E). miR-140-5p negatively targeted ALK5 and that ALK5 upregulated NOX2 in BV2 cells.

**EVs of MSCs transferred miR-140-5p into microglia**

Next, MSCs were extracted, followed by determination of classical surface markers of MSCs by flow cytometry (Figure 4A). The results manifested that CD13 (99.9%), CD90 (98.4%), and CD105 (100%) were highly expressed, while CD45 (0.4%) was low expressed. Additionally, the cells had osteogenic, adipogenic, and chondrogenic differentiation abilities (Figure 4B). These results indicated successful isolation of MSCs. TEM observation showed that isolated EVs were cup- or spherical-shaped (Figure 4C). Meanwhile, NTA results displayed that diameter of EVs was mainly distributed around 30-100 nm (Figure 4D). Also, CD9, CD63, and TSG101 expression was higher in EVs than
in cell lysate (Figure 4E), indicating successful extraction of EVs. Moreover, under the fluorescence microscope, it was observed that BV2 cells internalized MSC-EVs (Figure 4F). miR-140-5p expression was severely increased in BV2 cells by MSC-EV treatment, and it was more significantly elevated by EVs collected from miR-140-5p-transfected MSCs (Figure 4G, H). As exhibited in Figure 4I, red fluorescence existed and was strong in BV2 cells incubated with Cy3-miR-140-5p-EVs. Taken together, miR-140-5p could be transferred into microglia by MSC-EVs.

**MSC-EV containing miR-140-5p inhibited brain injury and microglial M1 activation in SAH mice**

Next, miR-140-5p mimic was transfected into MSCs, and then EVs with high or poor miR-140-5p expression (EVs-miR-140-5p mimic or EVs-miR-140-5p inhibitor) were extracted (Figure 5A). The SAH model following 1 h of construction was treated with MSC-EVs. Treatment with EVs promoted recovery of neurological function and reduced number of TUNEL-positive cells in cerebral cortex of SAH mice, which was strengthened by EVs-miR-140-5p. No alteration was noted in the neurological function and number of TUNEL-positive cells in SAH mice treated with EVs-miR-140-5p inhibitor relative to SAH mice (Figure 5B, C). Furthermore, ELISA exhibited that IL-6, IL-1β, and TNF-α expression in SAH mice was reduced by treatment with EVs or EVs-miR-140-5p, with more obvious decrease SAH mice treated with EVs-miR-140-5p. Additionally, no changes occurred in IL-6, IL-1β, and TNF-α expression in SAH mice treated with EVs-miR-140-5p inhibitor relative to SAH mice (Figure 5D). As described in Figure 5E, number of M1 microglia was declined while that of M2 microglia was increased in SAH mice treated with EVs, and treatment with EVs-miR-140-5p caused a more pronounced decrease in number of M1 microglia and a more pronounced increase in number of M2
microglia caused by EVs. In addition, number of M1 and M2 microglia showed no difference in SAH mice treated with EVs-miR-140-5p inhibitor relative to SAH mice. miR-140-5p expression increased and ALK5 and NOX2 expression decreased in SAH mice by EVs or EVs-miR-140-5p, with more marked changes in SAH mice treated with EVs-miR-140-5p. Moreover, miR-140-5p, ALK5 and NOX2 expression exhibited no change in SAH mice treated with EVs-miR-140-5p inhibitor relative to SAH mice (Figure 5F, G; Supplementary Figure 1F). MSC-EV-delivered miR-140-5p suppressed brain injury and microglial M1 activation in mice after SAH.

**miR-140-5p in MSC-EVs decreased ALK5 expression to inhibit brain injury and microglial M1 activation in mice after SAH**

To investigate the role of miR-140-5p in MSC-EVs in regulating microglial polarization after SAH, pretreatment with ALK5 was conducted at 48 h before SAH modeling and at 1 h after SAH modeling, treatment with MSC-derived EVs was performed in SAH mice. ELISA results manifested that the decline of IL-6, IL-1β, and TNF-α expression caused by EVs-miR-140-5p was reversed by overexpressing ALK5 in SAH mice (Figure 6A). Furthermore, treatment with EVs-miR-140-5p reduced number of M1 microglia yet increased number of M2 microglia in cerebral cortex of SAH mice, which was neutralized by further ALK5 overexpression (Figure 6B; Supplementary Figure 2D). Also, elevated miR-140-5p expression and decreased ALK5 and NOX2 expression were observed in SAH mice after treatment with EVs-miR-140-5p, which was negated by upregulating ALK5 (Figure 6C, D; Supplementary Figure 1G). MSC-EVs containing miR-140-5p could reduce microglial M1 activation and brain injury after SAH, but overexpression of ALK5 can reverse effect of MSC-EVs containing miR-140-5p.
Therefore, MSC-EVs containing miR-140-5p could repress microglial M1 activation after SAH by downregulating ALK5.

**Discussion**

SAH is a life-threatening stroke that occurs in younger people than other forms of stroke (Lawton and Vates, 2017). SAH can cause systemic inflammatory response syndrome and early or delayed brain injury (Macdonald and Schweizer, 2017). EV-derived miRs play a critical part in regulation of SAH caused by rupture of intracranial aneurysms (Liao et al., 2020). During the present investigation, we endeavored to study role of MSC-EV-shuttled miR-140-5p in the context of SAH. Our experimental data illustrated that MSC-derived EVs delivered miR-140-5p into microglia, downregulating ALK5 to decrease NOX2 expression, which ultimately suppressed M1 microglia activation and brain injury in SAH mice.

It is well-known that brain injury occurs immediately after SAH and results in neurological dysfunction and neuroinflammation (de Oliveira Manoel and Macdonald, 2018). A fundamental finding of our study was to identify overexpression of ALK5 in SAH mice and the inhibitory effect of ALK5 silencing on neurological dysfunction and neuroinflammation in mice after SAH. Similarly, ALK5 overexpression was detected in rats with cerebral ischemia/reperfusion injury, and ALK5 knockdown resulted in neurological function recovery of rats after cerebral ischemia (Zhang et al., 2019). Moreover, a prior study revealed that as the type I TGF-β receptor, ALK5 inhibition reduced NOX2 expression to promote neurological function recovery, brain infarction, and oxidative stress in rats with cerebral ischemia/reperfusion injury (Lou et al., 2018),
which supported our results that ALK5 silencing decreased NOX2 expression in SAH mice. Further experiments in our study elucidated that ALK5 silencing led to inhibition of M1 microglia activation in SAH mice by downregulating NOX2. It was well established that M1 microglia exacerbated tissue damage after SAH by secreting pro-inflammatory cytokines (Wei et al., 2017). As previously reported, NOX2 upregulation triggered M1-like microglia activation, neurodegeneration, and loss of neurological function after traumatic brain injury (Kumar et al., 2016). Also, present results concur with an earlier finding in SAH mice that upregulation of NOX2 in activated M1 microglia resulted in early brain injury (Pang et al., 2018). In conclusion, ALK5 silencing reduced M1 microglia activation to alleviate brain injury in SAH through NOX2 downregulation.

It is well acknowledged that miRs directly combine with mRNA of target genes to silence gene expression (Treiber et al., 2019). For instance, miR-140-5p negatively targeted Smad2, a key element downstream of TGF-β pathway, in colorectal cancer cells to repress cell proliferation and invasion (Zhai et al., 2015). Also, miR-140-5p directly targeted BMP2 to diminish osteogenic lineage commitment in undifferentiated human MSCs (Hwang et al., 2014). While in our study, ALK5 was a direct target gene of miR-140-5p in microglia. In the subsequent experiments, MSC-EVs delivered miR-140-5p to decrease M1 microglia activation and brain injury in SAH mice by decreasing ALK5 expression. Consistent with this, data obtained by Zhao et al. identified the neuroprotective effect of MSC-derived EVs on SAH-induced brain injury in rats (Zhao et al., 2019). Equally, intranasal administration of MSC-EVs could cause reduction of neuroinflammation induced by microglia in rats with perinatal brain injury (Thomi et al., 2019). The presence of miR-140-5p in MSC-EVs is reported in a prior study (Tao et al., 2017), which we confirm in this report. Interestingly, prior research elaborated that
ectopically expressed miR-140-5p diminished TLR4 expression to improve neurological function and to decrease apoptotic cell death and expression of inflammatory cytokines, thus alleviating brain injury and neuroinflammation of rats with intracerebral hemorrhage (Wang et al., 2019).

To conclude, our findings provide evidence for the suppressive effect of silencing ALK5 by MSC-EV-delivered miR-140-5p on M1 microglia activation and brain injury in SAH mice, the mechanism of which was dependent on NOX2 silencing (Figure 7). However, we only investigated the interaction between miR-140-5p and ALK5, which calls for further research about the specific mechanism between ALK5 and NOX2 for validation of the reported signaling axis. Although clinical therapeutic approaches involving miRNAs remain in their infancy, our present results are quite encouraging and suggest that MSC-EV-miR-140-5p can be targeted for the development of a novel treatment modality of SAH, providing a vista of the future research.
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Statement of Interest

The authors declare no conflict of interest.
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**Legends**

**Figure 1.** ALK5 silencing attenuates neurological dysfunction and neuroinflammation in SAH mice. Sham-operated mice were used as control, and SAH mice were treated or not treated with sh-NC or sh-ALK5. A, The expression of ALK5 determined by western blot analysis (n = 6). B, Quantification of the severity of subarachnoid hemorrhage evaluated by SAD grading (n = 6). C, Quantification of neurological score (n = 6). D, TUNEL/NeuN immunofluorescence staining (scale bar = 100 μm; DAPI: blue; TUNEL: green; NeuN: red) and the number of the TUNEL/NeuN positive cells (n = 6). E, The expression of IL-6, IL-1β, and TNF-α in serum detected by ELISA (n = 6). * p < 0.05 vs. sham-operated mice; # p < 0.05 vs. SAH mice treated with sh-NC. The data were measurement data, which were expressed by mean ± standard deviation. Comparison among multiple groups was analyzed by one-way variance of analysis.

**Figure 2.** ALK5 silencing reduces NOX2 expression to contribute to suppression of M1 microglia activation in SAH mice. A, Quantification of CD16-positive M1 microglia and CD206-positive M2 microglia. B, Western blot analysis of NOX2 protein expression in SAH mice after silencing ALK5. * p < 0.05 vs. sham-operated mice; # p < 0.05 vs. SAH mice treated with sh-NC. C, NOX2 knockdown efficiency confirmed by western blot analysis along with quantification of CD16-positive M1 microglia and CD206-positive M2 microglia. * p < 0.05 vs. WT mice. D, Western blot analysis of ALK5 protein expression in NOX2−/− mice pretreated with oe-ALK5 lentiviral vector. E, Quantification of CD16-positive M1 microglia and CD206-positive M2 microglia. n = 6. * p < 0.05 vs. NOX2−/− mice pretreated with vector. The data were measurement data, which were expressed by mean ± standard deviation. The comparison between the two groups was...
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**Figure 3.** ALK5 is negatively targeted by miR-140-5p in BV2 cells. A, TargetScan website predicting specific binding sites between ALK5 and miR-140-5p. B, The targeting relationship between ALK5 and miR-140-5p evaluated by dual luciferase reporter assay. C, RT-qPCR detection of the expression of miR-140-5p in BV2 cells transfected with miR-140-5p mimic. D, RT-qPCR detection of the mRNA expression of ALK5 and NOX2 in BV2 cells transfected with miR-140-5p mimic. E, Western blot analysis of the protein expression of ALK5 and NOX2 in BV2 cells transfected with miR-140-5p mimic. *\( p < 0.05 \) vs. BV2 cells transfected with mimic-NC. The data were measurement data, which were expressed by mean ± standard deviation. The comparison between the two groups was analyzed by unpaired \( t \)-test. The experiment was repeated three times.

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**Figure 7.** Mechanism. MSC-EVs transferred miR-140-5p into microglia, where miR-140-5p diminished the expression of ALK5, and thereby abolished the promoting effect of ALK5 on NOX2 expression, ultimately repressing M1 microglia activation and inflammatory response in SAH.
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