Exosome-mediated delivery of miR-486-3p alleviates neuroinflammation via SIRT2-mediated inhibition of mitophagy after subarachnoid hemorrhage

Supplemental information

1. Material and methods

Ethics and animals

Study participants were recruited from the Department of Neurosurgery, the Nanjing Drum Tower Hospital, the affiliated hospital of Nanjing University Medical School. The study was performed following the Declaration of Helsinki. All patients were approved by the Ethics Committee of the Nanjing Drum Tower Hospital (2021–313-07), and all mice were performed according to the guidelines of the National Institutes of Health on the care and use of animals. Healthy adult male C57BL/6J mice (8–10 weeks, 18–22 g) were purchased from the Animal Center of Nanjing University Model Animal Research Center, China. The mice were housed in a laboratory environment with temperature- and humidity-controlled animal quarters, a 12-h light/dark cycle, and free access to water and food.

SAH model

The SAH model was created as described previously[1]. Briefly, mice were anesthetized with 3% inhaled isoflurane and maintained with 1.5% during

surgery. The mouse was placed on a heating pad to maintain normal body temperature. The external carotid artery was ligated, slightly separated at the bifurcation, and the knot was left in place. The vessel was divided near the fracture end, and a prelabeled monofilament was inserted posteriorly from the external carotid artery through the internal carotid artery to the middle cerebral artery bifurcation. In the subarachnoid hemorrhage group, the monofilament was quickly inserted into the pointed nylon monofilament (6-0) with a slight breakthrough sensation and then pulled out; In the sham surgery group, preoperative preparation is the same as for SAH, the monofilament was quickly inserted into internal carotid with nylon monofilament (6-0) and then removed directly, not breakthrough vessel, then knots were tied to prevent bleeding. After surgery, the mice were placed in the animal postoperative care room until fully awakened; they were fed jelly to provide energy and water. Finally, a portion of the cerebrospinal fluid of the mice was first taken, and then the brain tissues were sampled and subjected to further analysis after SAH mice were sacrificed at the indicated time points. There were 130 mice recruited with a mortality rate of SAH mice was 6.9 %, and a total of 121 mice were eventually used for the following study. A schematic of experimental designs is given in supplementary Fig. S1.

Cell culture

Primary microglia were prepared as described previously[2]. Briefly,

cortical brain tissue from one-day-old suckling mice was collected and incubated with 0.25% trypsin for 10 min at 37 °C. After terminating with 10% FBS/DMEM (Gibco, USA), we filtered the supernatant of the tissue mixture using a 75-μm cell strainer. The cells were then resuspended in 10% FBS/DMEM and incubated in a cell culture incubator (5% CO_2, 37 °C) for seven days, and then the culture medium was replaced. At ten days, the upper mature microglia were collected. According to the manufacturer's instructions, Oxyhemoglobin (OxyHb) was produced using mouse hemoglobin (Sigma-Aldrich, USA). To simulate SAH in vitro, primary cultured microglia were incubated with OxyHb at 25 μM as described[3].

For neuron, cortical brain tissue from the fetal mice of pregnant mice were peeled off in layers, and the cortex was removed. After cortical digestion in 0.25 % trypsin for 5 min at 37 °C, medium with fetal bovine serum was added. We filtered the suspension of the tissue mixture using a 22-μm cell strainer. The supernatant was discarded and resuspended in DMEM. Cells were planted in poly-d-lysine-coated plates. The medium was replaced with a neural basal medium supplemented with glutamate and B27 after 4 hours. The neural basal medium was renewed every 2 days and continued 7–8 days of culture until the neurons were harvested. To simulate SAH in vitro, primary cultured microglia were incubated with OxyHb at 10 μM as described[4].

As previously described, bone marrow from adult male mice (6–8 weeks)

was mechanically harvested from femurs[5]. Cells were washed in phosphate-buffered saline (PBS) and suspended in 20% FBS/DMEM (Gibco, USA) with antibiotics (NCM Biotech, Suzhou). After three days, cells tightly adhered to the plastic flasks were P0 BM-MSCs. BM-MSCs were used within the eighth passage for exosomes collection.

Exosome isolation and identification

Exosome were purified from cell culture supernatants of BM-MSCs. Before collecting the culture medium, BM-MSCs were washed twice with PBS, and we replaced the culture medium with a serum-free medium. After incubation for 48 h, the supernatant was collected through sequential ultracentrifugation at 2,000 × *g* for 30 min, 10,000 × *g* for 60 min, and 200,000 × *g* for 90 min at 4 ºC using an L-90K ultracentrifuge (Beckman Coulter, USA). FBS was also spun before application to BM-MSC culture to avoid contamination due to FBS-derived exosome. The exosomes were washed once with PBS and resuspended for further characterization.

Well-established markers of purified exosome were verified by western blot analysis. The following antibodies were CD63, TSG101, Lamp2b and GM130. The size distribution of the precipitated particles was measured and analyzed using the Nanosight NS300 NTA System (Malvern Panalytical, Malvern, UK) according to the manufacturer's instructions. The morphology of exosome was examined using a transmission electron micrograph (TEM) (Hitachi HT7700, Tokyo, Japan). Briefly, 5 μL of the

previous eluate was extracted and diluted to 10 μL. Then, 10 μL of the sample was placed on a copper plate for one minute, and filter paper was used to remove the floating material. Subsequently, 10 μL of phosphotungsten acid was dropped on the copper plate for 1 min, and the floating matter was removed with filter paper. After drying for a few minutes, the samples were examined by TEM.

Concentration of exosome

A bicinchoninic acid (BCA) protein assay kit (Beyotime, P0010) was used to measure the concentration of exosome. Briefly, the previously isolated exosomes were melted at room temperature. Samples of exosome (2 μl) were loaded into each well of a 96-well plate, and 200 μl of BCA solution was added. After incubating for 30 min at 37 °C, optical density was measured at 570 nm on a microplate reader. The protein concentration of the sample was calculated according to the standard curve.

Lamp2b modification

The exosomal membrane protein gene Lamp2b was fused with the targeting peptide RVG and was amplified using 293T cell cDNA as a template, with the following primers: m-RVG-lamp2b-F and m-RVG-lamp2b-R (Supplemental Table S1). Nhe I and BamH I are endonuclease sites for plasmid construction with a pcDNA3.1(-) vector (Invitrogen, USA). BM-MSCs were electroporated with pcDNA3.1(-) -RVG-Lamp2b plasmid to produce RVG-positive exosome (Exo).

MiR-486-3p loading

Exosome (BCA Assay Kit, Beyotime) with a total protein content of 20 μg and 12 μl of miR-486-3p mimic (Exo/miR) or scrambled miRNA (Exo/Scr) (GenePharma, China) were added to 180 μl nucleofector buffer (Cell Line Nucleofector Kit V, Lonza) and electroporated in a Nucleofector IIs/2b device at 350 V and 150 μF. To remove the unincorporated free miR-486-3p, exosome was washed twice in PBS (4 °C) by sequential ultracentrifugation. Transfection efficiency was verified using RT-qPCR to measure miR-486-3p levels.

RNA isolation and RT-qPCR

Total RNA was extracted from cells using RNA isolation Total RNA Extraction Reagent (Vazyme, R401-01). Then, 500 ng of total RNA was reverse transcribed into cDNA using the HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, R211-01). Real-time fluorescent quantitative PCR was performed using HiScript II Q RT SuperMix for q-PCR (Vazyme, R222-01) under the following conditions: pre-denaturation at 94 °C for 30 sec, denaturation at 94 °C for 5 sec, and annealing at 60 °C for 30 sec, for a total of 40 cycles. mRNA was normalized using GAPDH as an internal reference (All mRNAs were seen in Supplemental Table S1). GAPDH ($2^{-\triangle\triangle Ct}$) was utilized as the reference gene for determining relative gene expressions. Results are based on at least three independent experiments.

Nuclear and cytoplasmic protein extraction

The nuclear and cytoplasmic proteins were extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, P0027). Briefly, cells were collected by centrifugation after washing with PBS, and cytoplasmic protein extraction reagent A containing PMSF was added. Vortex vigorously at high speed for 5 sec, followed by an ice bath for 10–15 minutes. Then, add cytoplasmic protein extraction reagent B, use Vortex at the highest speed for 5 sec, and keep it on ice for 1 minute. Vortex at high speed for 5 sec, and centrifuge at 12,000-16,000 × *g* for 5 min at 4°C. The supernatant is then transferred to obtain cytoplasmic proteins. For the precipitation obtained in the previous step, add PMSF-containing nuclear protein extraction reagent, Vortex at high speed for 15-30 sec, and Vortex at high speed for 30 min with intermittent ice bathing. Centrifuge at 12,000-16,000 × *g* for 10 min at 4 °C and transfer the supernatant to obtain nuclear protein. The obtained protein was used for subsequent experiments.

Western blotting

A BCA protein assay was extracted, and the protein from each group's ipsilateral cortical tissue and microglial cells was measured. Total and nuclear proteins were prepared using RIPA lysis buffer (P0013B, Beyotime, China). Equal amounts of protein were loaded and separated on 7.5–12.5%

sodium dodecyl sulfate-polyacrylamide gels, then electrophoresed and transferred to polyvinylidene difluoride membranes (Millipore, USA). The membranes were blocked with 5% skimmed milk for one hour, washed with Tris-buffered saline-Tween three times for 10 min, and incubated overnight with the following primary antibodies: CD63, TSG101, P62, Parkin, Pink1, PGC-1α, SIRT2, Histone H3, β-Tubulin, GAPDH, Lamp2, GM130, anti-acetyllysine mAb, and LC3 A/B (All primary antibodies were seen in Supplemental Table S2). After washing with Tris-buffered saline, the corresponding secondary antibodies were added and incubated for one hour. Protein signals were measured using Tanon 5260 Multi (Tanon, Shanghai) and quantified using Image J software (NIH, USA). All experiments were repeated three times.

Co-immunoprecipitation

Proteins were quantified to 2 mg/ml. We used two EP tubes for each sample to create the immunoprecipitation and IgG groups; one Eppendorf tube was used for the input group. Protein A/G PLUS–agarose beads (20 μl, Santa Cruz Biotechnology) were prepared with lysis buffer and then centrifuged to remove the supernatant of the beads. We added PGC-1α and IgG to the immunoprecipitation and IgG groups and incubated them on a rotating device overnight. All experiments were performed at 4 °C. We washed and resuspended samples the following day, then added

resuspended beads and rotated for two hours. The beads were centrifuged and washed three times. Finally, we added a 2X loading buffer and resuspended the samples. After boiling for five minutes, the material was centrifuged at 13000 rpm for one minute. Proteins were separated using sodium dodecyl sulfate-polyacrylamide electrophoresis and analyzed using western blotting. All experiments were repeated three times.

Enzyme-linked immunosorbent assay (ELISA)

At 24 h post-SAH, mice were anesthetized, and brain tissues were removed. The brain tissues were mechanically homogenized in 0.9% normal saline at 200 mg/ml and centrifuged at 12,000 × *g* for 10 min at 4 °C. The concentrations of IL-1β, IL-6, and TNF-α in brain tissue homogenates were quantified using ELISA kits for mice (ab241673, ab222503, and ab208348, Abcam, USA) following the manufacturer's instructions. The final concentration of cytokines was measured using optical density values.

Immunofluorescence staining and confocal imaging

Immunofluorescence staining was performed as previously described[6]. The mice were euthanized 24 hours after modeling and perfused intracardially with PBS, followed by 4% paraformaldehyde. The brains were fixed in 4% paraformaldehyde for 12 hours and then dehydrated in 15% and 30% gradient sucrose solutions for 24 hours. The brains were embedded and frozen in Tissue-Tek OCT. Coronal brain sections of 20 μm

were cut at 1.0 mm posterior to the bregma using a Leica NX50 cryostat. The sections were permeabilized, blocked, and incubated with primary antibodies overnight at 4 °C. The following antibodies were used: rabbit anti-CD86, rabbit anti-CD206, rabbit anti-Pink1, and rabbit anti-Parkin (all 1:200, Proteintech, China), rabbit anti-SIRT2, and goat anti-Iba1 (all 1:200, Abcam, USA). After washing three times in PBS, the tissue samples were incubated with the following secondary antibodies for one hour: Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor 594 goat anti-mouse IgG, Alexa Fluor 488 donkey anti-goat IgG or Alexa Fluor 594 donkey anti-rabbit IgG (Invitrogen, USA), then mounted with antifade mounting medium with DAPI (P0131, Beyotime, China) and fixed with coverslips for fluorescence photography. The photos were taken with a Leica microscope (LEICA DMi 8 Thunder). Mito Tracker@Red FM (M22425, Invitrogen, USA) was used to identify mitochondria, and Lyso-Tracker Red DND-99 (MX4317, Invitrogen, USA) was used to identify lysosomes. An Autophagy Staining Assay Kit with MDC (Beyotime, C3018S, China) was used to identify autophagosomes, and PKH26 (Sigma, PKH26GL-1KT) was used for exosome staining. All figures were taken with a laser scanning confocal microscope (LEICA DMi 8).

Brain water content

Brain edema was measured 24 hours after SAH, as previously described[7]. The brains were harvested and separated into ipsilateral and contralateral

hemispheres, and the olfactory bulbs, cerebellum, and brain stem were discarded, and then the brain was placed on a dry surface. Both hemispheres were wrapped in pre-weighed aluminum foil using an electronic analytic balance to obtain the total wet weight after incubating for 24h at 100 °C to obtain the dry weight. The percentage of water content was calculated as ([wet weight-dry weight] / wet weight) × 100%.

Blood-brain barrier permeability

BBB Permeability Operate according to the manufacturer's instructions. We injected mice with 5% Evans blue dye (E2129, 2%, 5 mL/Kg, Sigma-Aldrich) 24 h after SAH. One hour later, mice were perfused whole-body intracardiacly with PBS to clear the intravascular Evans blue dye. Mice were euthanized, and brain samples were harvested and homogenized in 50% trichloroacetic acid (TCA), after which the brain samples were centrifuged for 5 min at 1,000 × *g*. The supernatant was collected and mixed with ethanol and TCA at 4 °C overnight. The Evans blue dye in the supernatant was measured at 620 nm using a spectrofluorophotometer. Results are expressed as micrograms of Evans blue dye per gram of brain tissue.

Neurological and behavioral impairment

The modified Garcia score was used to evaluate the neurological behavioral impairment[8]. The Garcia test content 18-point scoring system includes spontaneous activity, symmetry of limb movement, forelimb

outstretching, climbing, body proprioception, and response to vibrissae touch. Higher scores indicate better function.

Fluoro-Jade C (FJC) staining

Neuronal degeneration was measured using a degenerated neuron kit (G3262, Solarbio, China). The previously obtained SAH mouse brain tissue was cryosectioned, rewarmed in distilled water for three minutes, and then dried at room temperature. The material was immersed in diluted solution A for 5 minutes, then transferred to 70% ethanol and washed with water. It was placed solution B for floating and washing with water. The liquid was evenly dropped on the treated brain slices and incubated in the dark for ten minutes, washed three times with distilled water, dried and clarified with xylene, and then sealed with neutral gum. Finally, the degeneration of neurons was observed under a fluorescent microscope (488 nm).

Flow cytometry (FCM)

FCM was performed to measure mitochondrial membrane potential and reactive oxygen species (ROS) levels. Mitochondrial membrane potential was measured with

tetramethylrhodamine and ethyl ester (TMRE, Thermofisher#T669, Suzhou). We diluted the stock solution with PBS and obtained 5 μM working solution. We centrifuged the cells, discarded the supernatants,

added a working solution, and incubated them in the dark. We centrifuged and discarded the supernatants and washed and resuspended cells in a serum-free cell culture medium. Finally, we subjected the cells to FCM using a 488 nm laser for excitation and emission at 575 nm.

Mitochondrial ROS levels were measured using MitoSOX (MitoSOX Red, HY-D1055, MCE). We diluted the stock solution with PBS for a 5 μM working solution. Cells were collected and washed, and then MitoSOX Red working solution was added and incubated in the dark. Cells were centrifuged, and we discarded the supernatants and washed them. We resuspended the cells and observed them using FCM (FITC channel).

Microglia apoptosis was measured using an Annexin V-FITC Apoptosis Detection Kit (C1062M, Beytoime). Briefly, we aspirated the upper cell culture medium, added PBS for washing, and added an appropriate amount of trypsin to digest the cells. The cells were incubated until they were detached by gentle pipetting at room temperature; then, we added the collected cell culture medium, gently pipetted the cells, transferred them to centrifuge tubes, spun, discarded the supernatant, collected, and resuspended the cells. This material was centrifuged; we discarded the supernatants, added Annexin V-FITC conjugate solution to resuspend the cells, added Annexin V-FITC, and mixed gently. Finally, we added propidium iodide, mixed gently, and incubated in the dark for 15 min at room temperature. Finally, apoptosis was measured using FCM.

Lentiviral transfection

We performed recombinant lentivirus infection experiments to construct SIRT2 and PGC-1α knockdown genes. The recombinant lentiviral vector was purchased from Shanghai Genechem (China). At 18–24 h before lentiviral transfection, the adherent cells were plated into 96-well plates at 5×10^4/well. The number of cells during lentiviral transfection was 1×10^5/well. After replacing the medium the following day, we added an appropriate amount of virus suspension. After incubating at 37 °C for 24–48 h, we added an appropriate amount of puromycin for screening. When the cell density reached 80%–90%, we collected the cells for RT-qPCR and western blot to measure the transfection efficiency. Successfully transfected genes were used for subsequent experimental studies.

Luciferase reporter assay

To determine whether miR-486-3p directly targeted SIRT2 mRNA, the luciferase reporter constructs were made by ligating the SIRT2 3'UTR fragments containing the predicted miR-486-3p binding sites. SIRT2 wild-type and its corresponding mutant were built into a pGL3-basic luciferase vector (Promega, USA) to generate the luciferase reporter plasmids. Cells were inoculated onto 24-well plates at an appropriate density and cultured at 37 °C. After 48 h of incubation and the cells reached about 70% confluence, they were co-transfected with luciferase reporter plasmids, miR-486-3p mimic, or NC mimic using Lipofectamine 2000 (Invitrogen,

USA) according to the manufacturer's instructions. Luciferase activity was examined using a dual-luciferase reporter assay kit (Promega Corporation, USA) following transfection for 24 h.

Molecular docking

This study downloaded the protein (SIRT2) structure file (PDB: AF-Q8IXJ6-F1) and protein (PGC-1α) structure file (PDB: AF-Q9UBK2-F1) from the PDB protein database.

HDOCK Server is used for flexible docking, generating 1000 different conformational orientations and obtaining the electrostatic and van der Waals interactions between the guest and the host, and calculating the Docking Score from this. By sorting the docking scores, the best-scoring conformation was obtained. Use Python (Version 3.7.7)-Pymol (Version 2.4.0) to process docking graphics and intermolecular spatial distances (distances less than 5Å indicate significant interaction forces). By removing intramolecular water molecules, protein subunits are color-coded, and visualization processing to finally generate publication-level images.

TEM

For microglia cells, we decanted the upper layer of cell culture medium and added a pre-fixative solution that had been pre-cooled to 4 °C. After fixing for 15 minutes, we gently scraped off the cells and centrifuged at 2000–3000 rpm for 10 minutes to collect the cell pellets (about the size of half a mung bean). For brain tissue, the fresh hemisphere basal cortex

samples from the exact location were harvested from the mice, with a size smaller than 1 mm^3. They were taken and stored in 2.5% glutaraldehyde (MilliporeSigma) for six hours at 4 °C. They were spread flat in 1% osmium tetroxide (MilliporeSigma) and dehydrated in ascending ethanol concentrations. They were treated with propylene oxide and impregnated in resin overnight at 25 °C. After embedding, they were cured at 60 °C for 48 h. We re-embedded the region of interest into the tip of the resin block and cut it to 70 nm using an ultramicrotome. Ultrathin sections were collected and examined under a TEM (Hitachi, Japan) at 80 kV[9].

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 Software (GraphPad Software, USA). All data were used Kolmogorov-Smirnov to tested for normal distribution. Those that conform to the normal distribution were presented as mean ± SD. Significance was assessed using a two-tailed Student's t-test to compare two groups. One-way analysis of variance followed by Tukey's post hoc test was used for three or more group comparisons. $P < 0.05$ was considered as statistically significant.

2. Results

Construction and delivery of engineering exosome

In our previous study, we performed high-throughput sequencing analysis on the serum of SAH and healthy control patients and showed that

11miNRAs were differentially expressed (GSE222980)[10]. Among them, the highly expressed miRNAs (miR-486-3p, miR-195-5p, miR-193b-3p), and the low expressed miRNAs (miR-410-3p, miR-136-3p, miR-369-3p) (Fig. S2A). Furthermore, q-PCR analysis showed that the levels of miR-486-3p and miR-193b-3p were higher in SAH patients than those in healthy controls; on the contrary, the levels of miR-410-3p and miR-369-3p were lower in the SAH patients. However, there was no difference in the miR-136-3p and miR-195-5p (Fig. S2B). Then, we used mRS[11] to evaluate its impact on the prognosis of SAH. The results showed that higher miR-410-3p and miR-369-3p indicate better outcomes. On the contrary, lower levels of miR-486-3p and miR-193b-3p indicate a better prognosis for patients (Fig. S2C). In order to further explore the impact of miRNAs after SAH, the SAH mice model was constructed. Cerebrospinal fluid (CSF) and brain tissue of mice were obtained after SAH at 24h for q-PCR analysis. The results were consistent with the serum of SAH patients: the levels of miR-410-3p and miR-369-3p decreased in CSF, while the levels of miR-486-3p and miR-193b-3p increased. Surprisingly, the opposite result was observed in the brain tissue of SAH mice, with increased levels of miR-410-3p and miR-369-3p, while the levels of miR-486-3p and miR-193b-3p were decreased. (Fig. S2D, S2E). In order to explore its impact on inflammation after SAH, we detected the levels of inflammatory factors (IL-1β, IL-6, TNF-α) respectively. The results

showed that the levels of IL-1β, IL-6, and TNF-α in the SAH group were higher than in the Sham group (Fig. S2F- S2H). Then, we tested neurological function, brain water content, and Evans blue level, respectively. The results showed that the neurological function of the SAH group was lower than that of the Sham group, and miR-486-3p was the lowest. Similar results were also observed in brain water content and Evans blue level. The brain water content and Evans blue level in the SAH group were significantly higher than those in the Sham group, and miR-486-3p was particularly obvious (Fig. S2I-2K). Because of this, we can improve the prognosis by increasing the levels of miR-486-3p in the brain tissue of mice.

Since miR-486-3p cannot freely pass through the BBB and has no targeted delivery, we constructed Exo with targeted delivery to pass through the BBB. To generate Exo, we fused the RVG peptide to Lamp2b and introduced the pcDNA3.1(-)-RVG-Lamp2b plasmid into BM-MSCs. Then, we loaded miR-486-3p mimic or Scr miRNA into Exo by electroporation. To determine whether the RVG-Lamp2b plasmid was successfully loaded into BM-MSCs, we measured the Lamp2b protein. Compared with BM-MSCs electroporated with pcDNA3.1(-) plasmid, western blotting analysis showed that RVG-Lamp2b-modified cells expressed higher levels of Lamp2b (Fig. S3A). Western blot analysis of exosomal marker Lamp2b, CD63, TSG101, and the Golgi marker GM130

confirmed the identity of the exosome and the inclusion of RVG-Lamp2b on exosome (Fig. S3B). Then, we observed modified exosome using TEM with the size of 90.09±34.84 nm (Fig. S3C, S3D). Next, we examined the efficacy of loading exosome with exogenous cargoes. The amount of encapsulated miR-486-3p was determined using q-PCR. The expression of miR-486-3p was significantly higher in exosome loaded with miR-486-3p mimic (RVG/miR) than in those loaded with scrambled miRNAs (RVG/Scr) (Fig. S3E). These findings suggest the efficient loading and stability of miR-486-3p in the modified exosome.

We established a SAH mouse model to investigate the potential of RVG-Exo to deliver miR-486-3p to the brain after SAH. We first injected fluorescein amidite (FAM)-labeled miR-486-3p intravenously at one day (D1) after SAH, and the mice were sacrificed after 2 hours (H2) to observed the distribution of FAM-labeled miR-486-3p in brain of mice. The result showed that there was barely FAM present after injection. This finding suggests that without exosome encapsulation, cells have difficulty endocytosing miRNA. Then, we injected unmodified Exo and RVG/Exo. We found more FAM-labeled Exo in the basal temporal lobe of mice in the RVG/Exo/miR group than in the unmodified Exo group. These findings suggest that compared with unmodified- Exo, RVG/Exo delivers miR-486-3p to temporal lobe hemorrhagic regions of the brain (Fig. S3F, S3G).

3. Supplemental Tables

Table S1. Sequence of primers, shRNAs used in the present study.

Primer name	Sequence (5′ → 3′)	
m-RVG-lamp2b	Forward	CACCATTTGGATGCCCGAGA
	Reverse	CCGTTGGATGCTCTCTTCCC
Sirt2	Forward	GCCTGGGTTCCCAAAAGGAG
	Reverse	GAGCGGAAGTCAGGGATACC
Sirt2 shRNA	Forward	GGAGCATGCCAACATAGATGC
	Reverse	GCATCTATGTTGGCATGCTCC
PGC-1α	Forward	TCTGAGTCTGTATGGAGTGACAT
	Reverse	CCAAGTCGTTCACATCTAGTTCA
PGC-1α shRNA	Forward	GGCAGUAGAUCCUCUUCAAGA
	Reverse	UUGAAGAGGAUCUACUGCCUG
IL-6	Forward	ACGTAGCTAGCTAGTCGGTATG
	Reverse	TCGTAGCTTGGCTAGTCGATCG
IL-1β	Forward	GCAGAGCACAAGCCTGTCTTCC
	Reverse	ACCTGTCTTGGCCGAGGACTAAG
TNF-α	Forward	ATGTCTCAGCCTCTTCTCATTC
	Reverse	GCTTGTCACTCGAATTTTGAGA
MiRNA-486-3p	Forward	GCGGGGCAGCTCAGTA
MiRNA-193b-3p	Forward	AACTGGCCCTCAAAGTCCCGCT
MiRNA-369-3p	Forward	accggccgcggAATAATACATGGTTGATCT TTT
MiRNA-410-3p	Forward	ccgcgggAATATAACACAGATGGCCTGT
MiRNA-136-3p	Forward	ccgcggCATCATCGTCTCAAATGAGTCT
MiRNA-195-3p	Forward	cgccggTAGCAGCACAGAAATATTGGC

Universal reverse primers	Reverse	CAGGTCCAGTTTTTTTTTTTTTTCGT
GAPDH	Forward	AGGTCGGTGTGAACGGATTTG
	Reverse	TGTAGACCATGTAGTTGAGGTCA

Table S2. Primary antibodies used in the study.

Antibody	Company (Cat. No.)	Working dilutions
CD63	CST (52090)	WB: 1/1000
TSG101	Proteintech (14497-1-AP)	WB: 1/2000
Lamp2	CST (49067)	WB: 1/1000
GM130	Proteintech (11308-1-AP)	WB: 1/5000
P62	HuaAn (HA721171)	WB: 1/5000
Parkin	Proteintech (14060-1-AP)	WB: 1/2000; IF: 1/200
Pink1	Proteintech (23274-1-AP)	WB: 1/1000; IF: 1/200
SIRT2	Proteintech (25682-1-AP)	WB: 1/5000
SIRT2	Abcam (ab134171)	IF: 1/200
CD86	Proteintech (13395-1-AP)	IF: 1/200
CD206	Proteintech (18704-1-AP)	IF: 1/200
Iba1	Abcam (ab178846)	IF: 1/200
PGC-1α	Proteintech (66369-1-AP)	WB: 1/5000
Histone H3	HuaAn (A11-D7)	WB: 1/10000
β-Tubulin	Proteintech (10094-1-AP)	WB: 1/5000
GAPDH	Proteintech (10494-1-AP)	WB: 1/10000
LC3 A/B	CST (12741)	WB: 1/1000
anti-acetyllysine mAb	PTMBIO (PTM-101)	WB: 1/1000

4. Supplemental Figures

Figure S1. The schematic of experimental designs in this study.

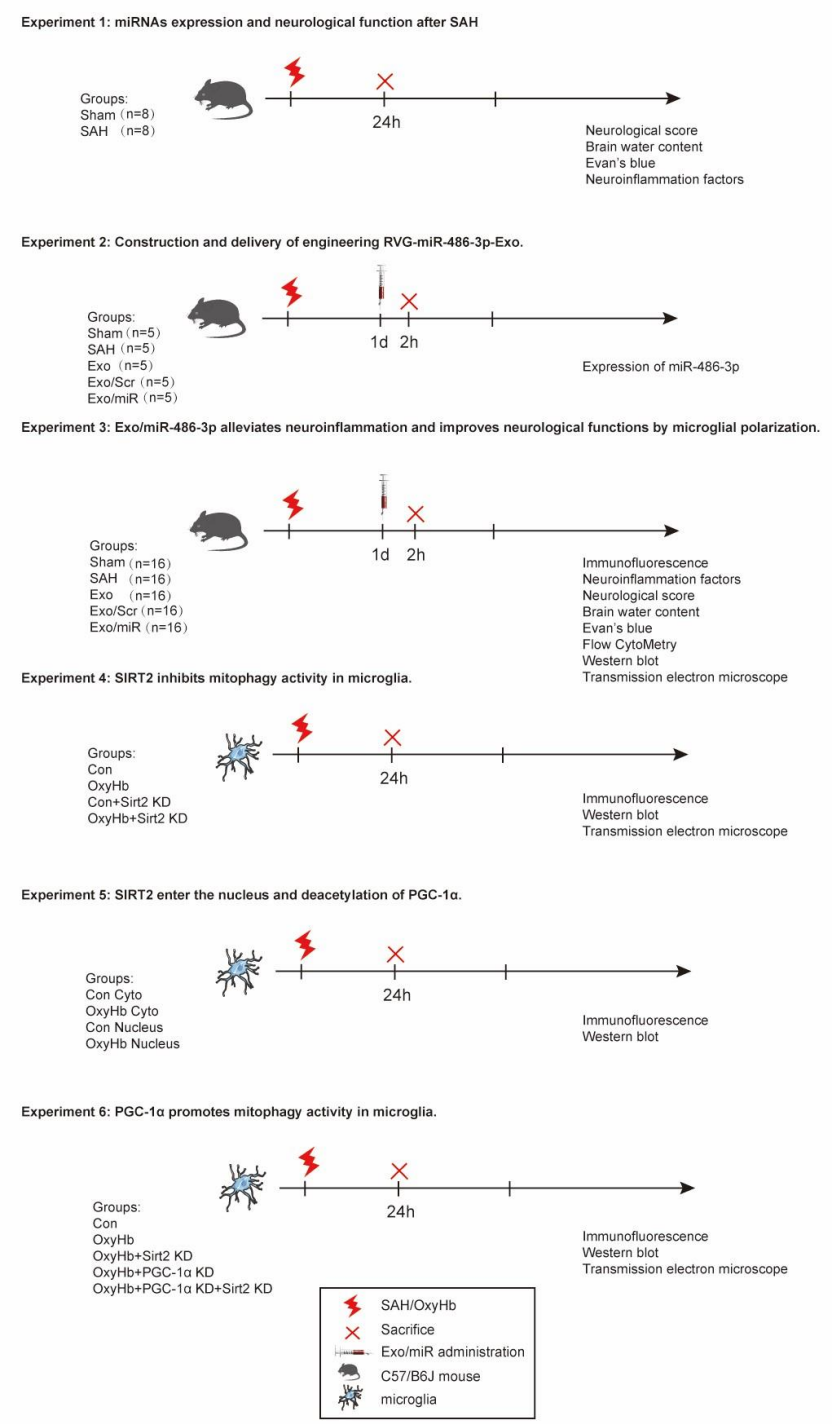


Figure S2. The level of miRNAs in patients/mice after SAH. (A-B). The different miRNAs of next-generation sequencing were validated in SAH patients and healthy controls. **(C).** Relative levels of miRNAs in relation to clinical outcome in SAH patients and healthy controls. **(D-E).** Relative levels of miRNAs in CSF(D) and brain(E) of SAH mice. **(F-H).** the levels of inflammatory factors (IL-1β, IL-6, TNF-α) by ELISA. (**I)**, Neurological score, (**J),** brain water content and (**K),** Evan's blue in each group at 24h after SAH.

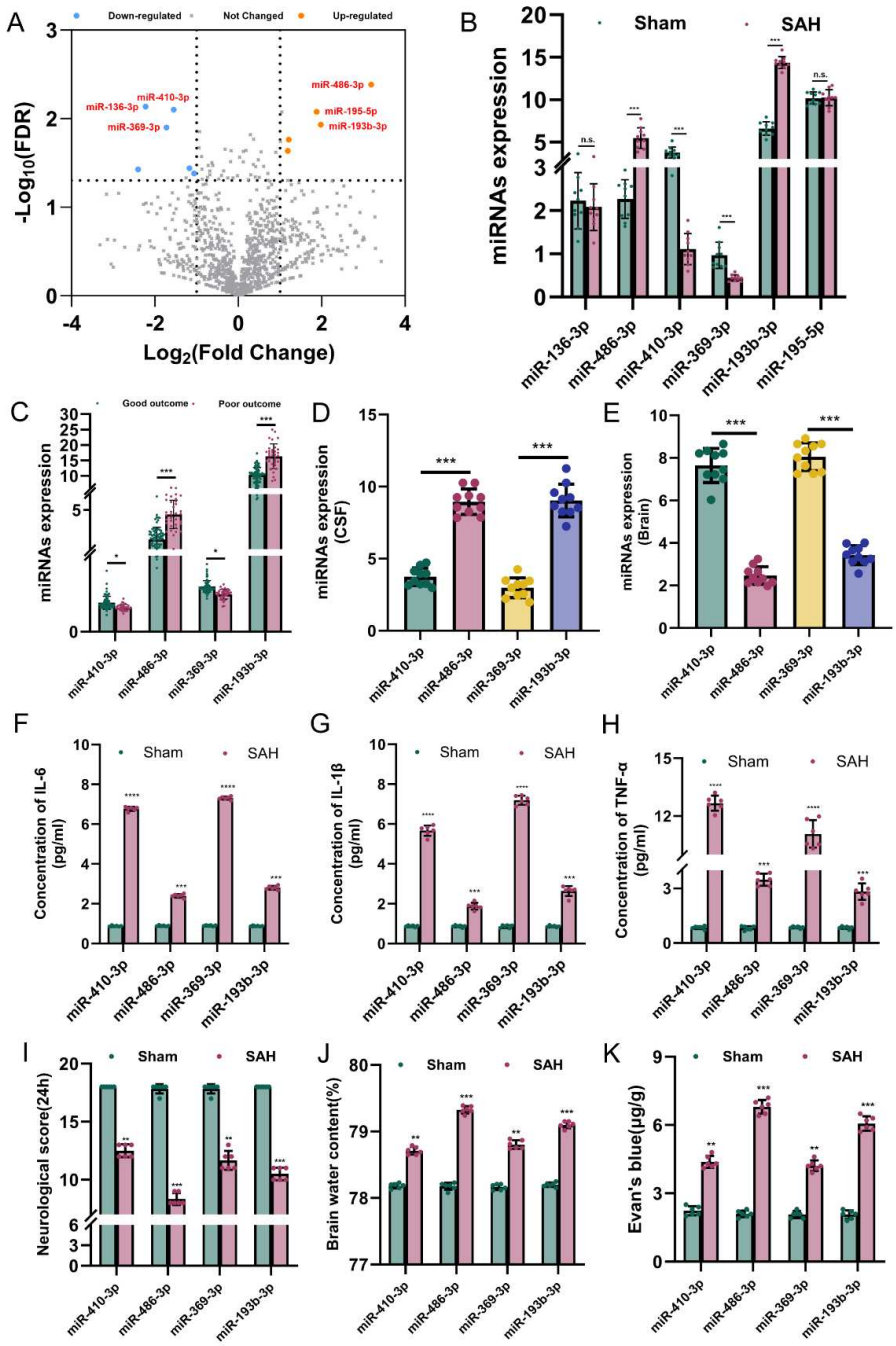

A
Down-regulated
Not Changed
Up-regulated
-Log10(FDR)
Log2(Fold Change)
miR-136-3p
miR-410-3p
miR-369-3p
miR-486-3p
miR-195-5p
miR-193b-3p
B
Sham
SAH
miRNAs expression
n.s.
miR-136-3p
miR-486-3p
miR-410-3p
miR-369-3p
miR-193b-3p
miR-195-5p
C
Good outcome
Poor outcome
miRNAs expression
D
miRNAs expression (CSF)
E
miRNAs expression (Brain)
F
Concentration of IL-6 (pg/ml)
G
Concentration of IL-1β (pg/ml)
H
Concentration of TNF-α (pg/ml)
I
Neurological score(24h)
J
Brain water content(%)
K
Evan's blue(μg/g)

Fig. S3. Construction and delivery of engineering Exo/miR. (A), Western blotting of Lamp2b in electoporated BM-MSCs. (**B),** Western blot analysis of Lamp2b, GM130, CD63 and TSG101 in RVG-Lamp2b-modified exosome and the parental cells. (**C**), RVG-Exo were measured by NTA. (**D),** Transmission electron microscopy of RVG-Exo isolated from modified exosome. Scale bar, 100 nm. (**E),** Expression levels of miR-486-3p quantified in exosome in RVG/Scr vs RVG/Exo. (**F-G),** Schematic diagram of modified exosome delivered to brain and immunofluorescence images of miR-486-3p deliver to brain tissues of mice after SAH in Scr/miR, Exo/miR and RVG/Exo/miR group. Scale bar: 50 μm. **** $P < 0.0001$.

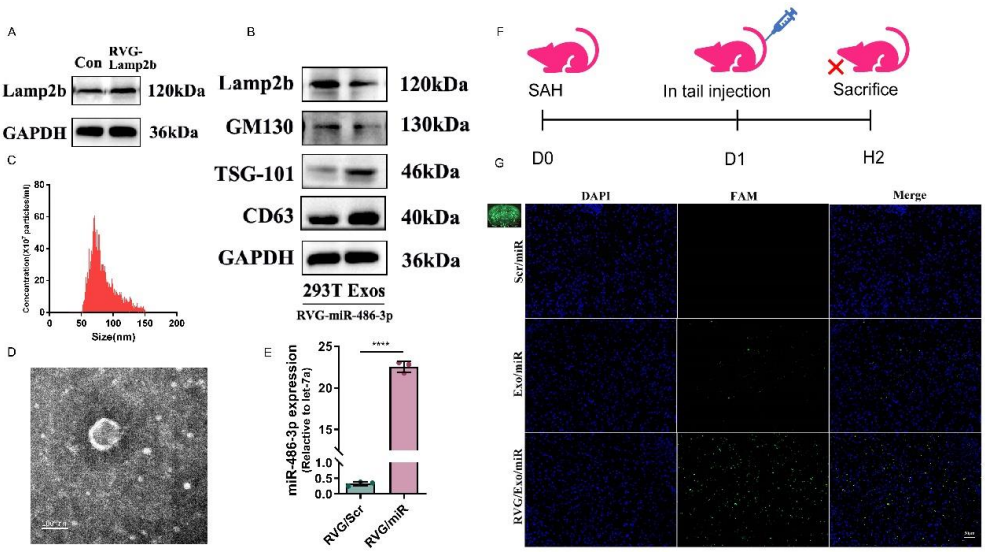


Figure S4. The knockdown efficiency of sirt2 shRNA

(A). q-PCR analyses of sirt2 shRNA expression in microglia. **(B-C).**

Western blot and quantitative analysis of SIRT2 shRNA in microglia.

*** P < 0.001, n.s., no significant.

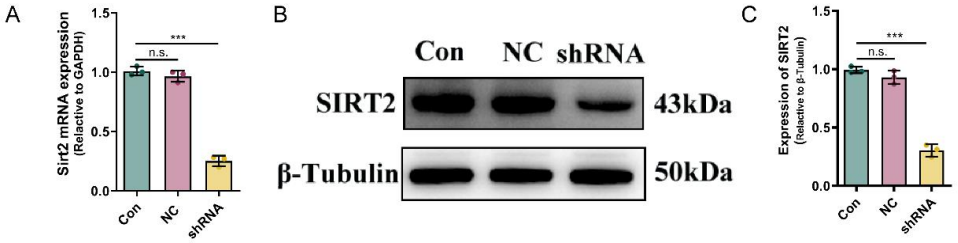
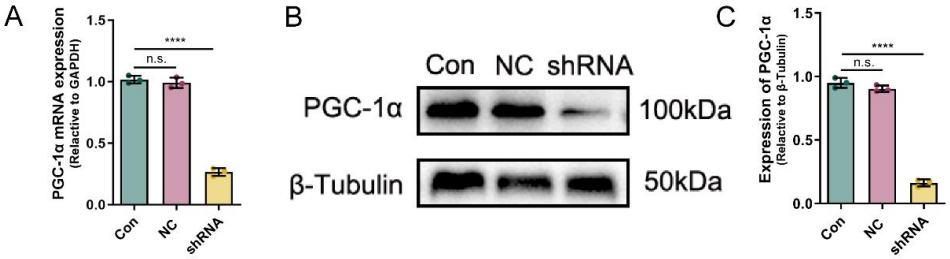


Figure S5. The knockdown efficiency of PGC-1α shRNA

(A). q-PCR analyses of PGC-1α shRNA expression in microglia. **(B-C).** Western blot and quantitative analysis of PGC-1α shRNA in microglia.

**** P < 0.0001, n.s., no significant.



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