Exosomes derived from bone marrow mesenchymal stem cells promote angiogenesis via transfer of miR-21-5p after cerebral ischemia in mice

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Research Article

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Abstract

Background

Mesenchymal stem cells (MSCs) transplantation is a potential clinical therapy for cerebral ischemia. The therapeutic effects of MSCs primarily depends on the paracrine action by releasing exosomes (Exos). Exosomes derived from bone marrow mesenchymal stem cells (BMSC-Exos) could modulate target cell functions by transferring microRNAs (miRs) cargo. In this study, we aimed to investigate whether BMSC-Exos could promote angiogenesis via transfer of miR-21-5p after cerebral ischemia.

Methods

BMSC-Exos were isolated from conditioned medium of BMSCs by differential ultracentrifugation, and confirmed by transmission electron microscopy, nanoparticle tracking analysis, and western blot analysis. In mice with middle cerebral artery occlusion (MCAO), the neurological function was evaluated by Zea Longa's method, and the infarct volume and microvessel density were detected by TTC staining and vWF immunofluorescence staining, respectively. The proangiogenic effects of BMSC-Exos were assessed via proliferation, migration, and tube formation of human umbilical vein endothelial cells (HUVECs) in vitro assays. The miR-21-5p expression was detected by qRT-PCR. The expression levels of VEGF, VEGFR2, Ang-1, and Tie-2 were determined by western blot.

Results

BMSC-Exos significantly improved neurological function and reduced infract volume after cerebral ischemia. Moreover, BMSC-Exos significantly upregulated the microvessel density and the expression levels of proangiogenic proteins VEGF, VEGFR2, Ang-1 and Tie-2 in the ischemic boundary region. MiR-21-5p expression was also dramatically increased after cerebral ischemia. In vitro assays revealed that BMSC-Exos enhanced HUVECs functions including proliferation, migration and tube formation, as well as increasing the expression of VEGF and VEGFR2. However, these proangiogenic effects of BMSC-Exos on HUVECs were reversed by miR-21-5p inhibitor.

Conclusion

Our study indicated that BMSC-Exos could promote angiogenesis and neurological function recovery via transfer of miR-21-5p. Therefore, the application of miR-21-5p-loaded BMSC-Exos might be an attractive treatment strategy of cerebral ischemia.

Introduction
Stroke is one of the leading causes of death and disability worldwide [1]. Up to now, the only recommended drug for treatment of acute cerebral ischemia is tissue-type plasminogen activator (tPA), which works as a thrombolytic agent. However, the narrow therapeutic time window and potentially fatal hemorrhage severely limited the clinical application of tPA [2]. Therefore, novel effective strategies designed to improve functional recovery after ischemic stroke are urgently needed. A growing number of researches have indicated that stem cell-based therapy represents a new approach for the treatment of ischemic stroke [3-5]. Mesenchymal stem cells (MSCs) can be obtained easily and expanded rapidly in vitro, which make it an ideal candidate for cell-based therapy [6]. A variety of studies have demonstrated that transplanted MSCs could promote neurogenesis and angiogenesis, thus facilitate the recovery from stroke [7, 8]. As for the underlying mechanism of these functions, it was initially believed that MSCs homed to ischemic tissues and differentiated to replace injured cells [9, 10]. Nevertheless, following researches reported that the engraftment and differentiation of MSCs into impaired sites are at a poor level and transient [11, 12]. Until now, it is proposed that MSCs exert their beneficial effects mainly by secreting paracrine factors, such as trophic factors and extracellular microvesicles (EVs) [13, 14].

Exosomes (Exos) are small EVs sized approximately 30-150 nm in diameter [15]. They are secreted by a diversity of cell types, and contain biological molecules, including proteins, lipids, mRNAs and microRNAs (miRs) [16]. The beneficial effects of MSCs-derived Exos on stroke have been particularly studied, but the mechanisms of action are still not fully clarified [17, 18]. Recent studies have shown that Exos transfer miRs to the recipient cells in the brain to affect the nervous and vascular systems, and ultimately alleviate cerebral ischemic injury [19-21].

MiRs are small single-stranded noncoding RNAs that bind to target mRNAs and suppress proteins expression by inhibiting translation or degrading mRNAs [22]. They are engaged in many physiological and pathological processes, such as cell proliferation, migration, differentiation, metabolism and apoptosis [23, 24]. Increasing evidence has demonstrated that miRs are important regulators of angiogenesis. MiR-21-5p notably promoted local neovascularization in myocardial infarction model [25]. Exos derived from endothelial progenitor cell can promote endothelial cell repair by transferring miR-21-5p [26]. MSCs could promote ischemic tissue repair and angiogenesis of diabetic foot by exosomal miR-21-5p [27]. Recent studies indicated that miR-21-5p was abundantly expressed in BMSC-Exos [28]. However, it is not clear whether BMSC-Exos promote angiogenesis after cerebral ischemia through transfer of miR-21-5p.

In this study, we firstly studied the putative beneficial effects of BMSC-Exos on the neurological function and angiogenesis with cerebral ischemia, and then further confirmed whether these proangiogenic effects are related to the miR-21-5p transferred by BMSC-Exos.

Materials And Methods

Animals
Male Sprague-Dawley rats weighing 80-100 g (3 weeks old) and male ICR mice weighing 25-30 g (8-10 weeks old) were obtained from SIPPR/BK Laboratory Animal (Shanghai, China). The animals were kept at stable temperature (22 ± 2 °C) and humidity (60 ± 5%) condition, with 12-h light/dark cycle and free access to water and food. All animal care and experimental procedures were approved by the Zhejiang Chinese Medical University Animal Welfare Ethics Committee (reference number: ZSLL-2017-058), and performed in accordance with the NIH guidelines for the Care and Use of Laboratory Animals [29].

Cell culture

BMSCs were isolated based on our previously described method [30]. Briefly, the bone marrow was flushed with prechilled DMEM/F12 containing 1% (v/v) penicillin and streptomycin (Haotian Biological Technology, Hangzhou, China) from the femurs and tibias of rats. Then, centrifuged the bone marrow aspirates and suspended the cells in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, MD, USA). The medium was replaced 2 days after and every 3 days thereafter. The cells were passaged when 80-90% confluence was reached with a ratio of 1:2. The passage 3-4 BMSCs were used for the following experiments. To identify the BMSCs, cells were detected by flow cytometry using specific antibodies against cell surface markers, including CD34 (Santa Cruz, CA, USA), CD29, CD45 and CD90 (Biolegend, San Diego, CA, USA).

Human umbilical vein endothelial cells (HUVECs) obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 medium (Hyclone, UT, USA) containing 10% FBS and 1% antibiotics. Humidified condition at 37 °C with 5% CO₂ was maintained. HUVECs were passaged every two or three days.

BMSC-Exos isolation, purification and identification

BMSC-Exos were isolated and purified by differential ultracentrifugation method [31]. Briefly, upon reaching 70-80% confluence, BMSCs were rinsed three times with PBS and cultured in fresh medium containing 10% Exos-free FBS medium (SBI Biosciences, CA, USA). The supernatants were collected after an additional 48 h incubation, and sequentially centrifuged at 300 × g and 2000 × g for 10 min to remove the dead cells. Then, the supernatant was centrifuged at 10,000 × g for 30 min at 4°C to clear the residual cellular debris and filtered with a 0.22-μm filter (Millipore, MA, USA). Afterwards, the filtrates were ultracentrifuged at 4°C and 100,000 × g for 2 h, followed by washing with PBS and ultracentrifuged at 100,000 × g for 2 h. At last, the pelleted Exos were resuspended in 100 μL PBS and quantified by total proteins using a Micro BCA Protein Assay kit (Thermo Scientific, IL, USA), then stored at -80°C.

For identification of Exos, the morphology, particle concentration, size distribution, and specific surface markers (CD9, CD63 and TSG101) of isolated Exos were detected by transmission electron microscope (TEM, Hitachi, Japan), nanoparticle tracking analysis (NTA), and western blot analysis respectively.

Focal cerebral ischemia model in mice
Focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) as previously described [32]. Briefly, male ICR mice were intraperitoneally anesthetized with 10% chloral hydrate at a final dose of 350 mg/kg. Then, a tip blunted and poly-L-lysine coated 6-0 nylon monofilament suture was advanced approximately 10 mm distal to the right carotid bifurcation to occlude the origin of the MCA. After 60 min of ischemia, the suture was carefully and slowly withdrawn. Sham-operated mice received identical surgery without inserting a suture. During the surgery, the rectal temperature of mice was maintained at 37°C with a homeothermic pad.

**BMSC-Exos administration and BrdU labeling**

To evaluate the therapeutic effects of BMSC-Exos, mice were allocated to four cohorts: sham operation group, MCAO group, 25 μg BMSC-Exos group and 50 μg BMSC-Exos group, and then were administered with Exos in 100 μL PBS or PBS alone by tail vein injection at 24 h after ischemia. To observe cell proliferation, mice were administered 50 mg/kg of 5-bromo-2-deoxyuridine (BrdU, Sigma-Aldrich, MO, USA) by initial intraperitoneal injection 24 h after ischemia, followed by daily consecutive injection for up to 14 days.

**Neurological function evaluation**

The neurological deficit score was evaluated 1, 3, 7, and 14 days after stroke according to the Zea Longa score [33]: 0, no deficit; 1, failure to fully extend left forepaw; 2, circling to the left; 3, paresis to the left; 4, depressed level of consciousness and no spontaneous walking. The corner test was carried out as previously described [34]. Briefly, two 30 cm×20 cm×1 cm boards were attached to each other at an angle of 30° and with a small opening between the two boards. A mouse was placed into the central square facing the corner. When both sides of the vibrissae were stimulated by boards, the mouse then reared forward and upward, after which it turned back to face the open end. Each mouse was tested for ten trials, and the selected turning sides were recorded.

**Infarct volume assessment**

Mice were euthanized by overdose anesthesia with 10% chloral hydrate, and brains were moved and frozen immediately at -20°C for approximately 5 min, and then dissected into 1 mm-thick coronal slices. The slices were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) for 15 min, and fixed with 4% paraformaldehyde for 24 h. The infarct volume was evaluated by Image J software.

**Immunofluorescence staining**

Mice were transcardially perfused with normal saline followed by 4% paraformaldehyde solution for 10 min at 14 days after MCAO. Brains were fixed overnight at 4°C, and then soaked in 30% sucrose solution. The brains were frozen and cut into 10-μm-thick frozen sections (Leica, Wetzlar, Germany). BrdU/von Willebrand factor (vWF) was detected by double immunofluorescence staining as described in our previous study [30].
BMSC-Exos uptake by HUVECs

To label Exos with green fluorescent dye, Exos were first resuspended with the PKH67 dye (Sigma-Aldrich, Munich, Germany) and incubated for 5 min, then terminated by 2% bovine serum albumin. Next, Exos were ultracentrifuged at 100,000 × g for 1 h to clear unbound dyes. HUVECs were incubated with PKH67-labelled Exos for 12 h followed by 4% paraformaldehyde fixation for 15 min and 4',6-diamidino-2-phenylindole (DAPI) stain for 5 min. The internalization of PKH67-labeled Exos by HUVECs was observed using a fluorescence microscope (Leica, Wetzlar, Germany).

MiR-21-5p inhibitor transfection

The miR-21-5p inhibitor and the negative control (NC) were synthesized by RiboBio (Guangzhou Ribobio, Guangzhou, China). BMSCs at 80% confluence were transfected with 100 nM miR-21-5p inhibitor or NC, which were performed using Lipofectamine 2000 and Opti-MEM medium (Invitrogen, CA, USA) according to the manufacturer’s protocol. After 6 h, the transfection mixture was replaced by DMEM/F12 containing 10% exosome-free FBS. Conditioned medium of transfected cells was collected and centrifuged as described above.

MTT assay

The proliferation of HUVECs was measured using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Beyotime, Shanghai, China). HUVECs plated on 96-well plates (1×10^4 cells/well) were cocultured with Exos (25 and 50 µg/mL) for 12, 24, 36, and 48 hours, and then 10 µL of MTT tetrazolium salt solution (5 mg/mL) was added into each well. After incubation for another 4 h, the formazan crystals were dissolved by adding 150 µL of dimethyl sulfoxide (Sigma, MO, USA). The optical density (OD) value of each well was measured at 490 nm by microplate reader (Tecan Austria GmbH, Grodig, Austria). Each experimental group was performed in six replicate wells.

Scratch wound healing assay

HUVECs were seeded into 6-well plates (5×10^5 cells/well) for confluence, followed by scratching with 200 µL pipette tip when reaching 90% confluence. Then, 2 mL of serum-free RPMI-1640 medium supplemented with Exos was added to each well. Images were captured at 0 h and 24 h after wounding. The migration rate (%) was calculated as follows: migration rate (%) = (initial wound area (t=0 h) – residual area (t=24 h)) / initial wound area (t=0 h)×100%. Each experiment was repeated three times.

Transwell migration assay

Transwell migration assay was carried out using 24-well chambers (8 µm, Corning, NY, USA). 500 µL of the RPMI-1640 containing 1% FBS was added into the lower chamber, and HUVECs (6×10^4 cells/well) suspended in 100 µL FBS-free medium were seeded in the upper chamber in the presence or absence of Exos. After 8 h for migration, nonmigratory cells were removed from the top of the insert membrane using humidified cotton swabs. The migrated cells at the bottom surface of membrane were fixed in 4%
paraformaldehyde and stained with 0.1% crystal violet. The migrated cells were imaged and counted at 5 random fields. Three duplicates were set in each group.

**Tube formation assay**

After thawed overnight at 4 °C, 50 μL per well matrigel matrix (BD Biosciences, CA, USA) was added into precooled 96-well plates and incubated at 37°C to polymerize for 30 min. Next, HUVECs (2 × 10⁴ cells/well) in FBS-free RPMI-1640 containing Exos were seeded onto matrigel-coated plates. Capillary-like tubular structures were captured after 6 h incubation. The total tube lengths from five random microscopic fields were calculated using Angiogenesis Analyzer Image J software.

**Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)**

Total RNA from brain tissues of ischemic boundary region or cells was extracted using miRNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was produced from the total RNA using Mir-X™ miRNA First-strand Synthesis Kit (TaKaRa, Dalian, China). Subsequently, the product from reverse transcription was amplified with the SYBR Premix Ex Taq Kit (TaKaRa, Dalian, China) on an iQ5 real-time PCR detection system (Bio-Rad, CA, USA). Relative expression levels of miRNA were calculated by the 2⁻ΔΔCt and were normalized to U6. Each sample repeated 3 times and at least three samples obtained from independent experiments were examined. All primers used in this study are listed in Table 1.

**Table 1** Primer sequences for qRT-PCR

| Primers   | Sequences                      |
|-----------|--------------------------------|
| miR-21-5p | 5’-CCGCGTAGCTTATCACGACTCAGACTGATGTTGA -3’ |
| miR-22-3p | 5’- CGAAGCTGCCAGTTGAAGAACTGT -3’          |
| miR-486   | 5’- TCCTGTACTGAGCTGCCCC -3’              |
| let-7i-5p | 5’- GCGTGAGGTAGTAGTTTGTGCTGTT -3’        |

Note: The forward and reverse primers of U6, and the mRQ 3’Primer used as the universal reverse primer for above miRs, were supplied in the Mir-X miRNA First-strand Synthesis kit (Cat No. 638313)

**Western blot analysis**

Total protein was extracted with RIPA lysis buffer containing protease inhibitor PMSF (Beyotime, Shanghai, China), and quantified using BCA protein assay kit (Beyotime, Shanghai, China). Firstly, equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to PVDF membranes (Millpore, CA, USA). After being blocked with 5% skim milk in TBST, the membranes were incubated with the following primary antibodies overnight at 4°C: CD9 (1:1000; Bioworld, MN, USA), CD63 (1:1000; Bioworld, MN, USA), TSG101 (1:1000; Abcam, MA, USA), VEGF (1:500; Santa Cruz, CA, USA), VEGFR (1:500; Abcam, MA, USA), Ang-1 (1:1000; Santa Cruz, CA, USA), Tie-2
(1:1000; Santa Cruz, CA, USA) and GAPDH (1:1000; Santa Cruz, CA, USA), followed by incubation with corresponding secondary antibodies at room temperature for 1 h. Signals were visualized by enhanced chemiluminescence detection kit (Millipore, CA, USA). Relative expression levels were normalized to levels of GAPDH.

Statistical analysis

Data were analyzed by SPSS software (version 25.0, SPSS, IL, USA) and presented as mean ± standard deviation (SD). Neurological deficit and corner test data were analyzed by nonparametric Kruskal-Wallis H test. One-way analysis of variance (ANOVA) (Student-Newman-Keuls) statistical differences between groups was used for analyzing all other data. P<0.05 was considered statistically significant.

Results

Characterization of BMSCs and BMSC-Exos

The primary BMSCs (P0) initially displayed short bar shapes, while BMSCs at passage 3 (P3) exhibited a typical spindle-shaped morphology (Fig. 1a). To characterize the phenotype of BMSCs, the BMSCs surface markers were analyzed by flow cytometry. The results showed that these cells were negative for CD34 (1.44%) and CD45 (0.30%) (hematopoietic cell specific markers), whereas strongly positive for CD29 (99.83%) and CD90 (99.88%) (mesenchymal stem-cell specific markers) (Fig. 1b).

As presented in Fig. 2a, the BMSC-Exos had a typical spherical or cup-shaped morphology. NTA revealed a bell-shaped curve of BMSC-Exos size distribution with a peak at approximately 108.4 nm, and an average concentration of the particles was $4.8 \times 10^7$ particles/ml (Fig. 2b). Additionally, the isolated BMSC-Exos expressed exosomal markers CD9, CD63 and TSG101 without the expression of non-exosome marker protein GAPDH (Fig. 2c). These properties indicated that the BMSC-derived particles were Exos.

BMSC-Exos ameliorated ischemic brain injury in mice

To determine whether BMSC-Exos improved neurological function recovery, the neurological deficit score and the corner test were performed at 1, 3, 7 and 14 days after MCAO in mice. The results demonstrated that BMSC-Exos decreased the neurological deficit score and right-turn number in dose-related manner (Fig. 3a-b). Results of TTC staining showed that BMSC-Exos significantly reduced infarction volume on 3 days after ischemia. At 14 days after ischemia, apparent atrophy of the ischemic hemisphere was observed in all groups of mice, except the sham-operated group. Moreover, the cerebral atrophy in the BMSC-Exos group was markedly reduced compared to the MCAO group. In addition, compared with the 25 μg BMSC-Exos group, the cerebral atrophy was significantly reduced in the 50 μg BMSC-Exos group (Fig. 3c-d).

BMSC-Exos promoted angiogenesis in the ischemic boundary region
Microvessel density was estimated by BrdU/vWF immunofluorescent staining at 14 days after MCAO. Significantly increased number of BrdU+/vWF+ cells was observed in the MCAO and BMSC-Exos groups compared to the sham group. Furthermore, microvessel density in the BMSC-Exos group increased more significantly than which in MCAO group (Fig. 4a-b).

**BMSC-Exos increased VEGF/VEGFR and Ang-1/Tie2 protein expression after ischemia in mice**

To test whether BMSC-Exos promote angiogenesis via upregulating the expression of VEGF, VEGFR2, Ang-1 and Tie2, the expression levels of these proteins were detected by western blot analysis. Compared with the sham group, the expression levels of VEGF, VEGFR2, Ang-1 and Tie2 were increased in the MCAO group and BMSC-Exos group. Furthermore, the expression levels of these proteins were increased more obviously in BMSC-Exos group compared to the MCAO group (Fig. 4c-d).

**BMSC-Exos increased miR-21-5p expression after ischemia in mice**

To study the changes of specific miRs expression levels after stroke, the expression of several candidate miRs, including Let-7i-5p, miR-21-5p, miR-22-3p and miR-486 were detected by qRT-PCR at 14 days after MCAO. The expression levels of these miRs were increased in the BMSC-Exos group compared to the MCAO group. In particular, miR-21-5p expression levels increased approximately 22-fold (Fig. 5a-d).

**HUVECs uptake BMSC-Exos**

To confirm that BMSC-Exos could be internalized into HUVECs, BMSC-Exos were labeled with PKH67 and then were added to HUVECs for 12 h incubation. Fluorescence images showed that PKH67 fluorescence were incorporated into the cytoplasm of HUVECs (Fig. 6a). This result revealed that our purified BMSC-Exos had been taken up and transferred to HUVECs.

**BMSC-Exos promoted HUVECs angiogenesis by transferring miR-21-5p**

Since endothelial cell proliferation, migration, and sprouting of are critical for angiogenesis, the effects of BMSC-Exos on HUVECs proliferation, migration and tube formation were studied. MTT assay, scratch wound healing assay, transwell assay and tube formation assay indicated that BMSC-Exos can enhance the above-mentioned capability of HUVECs (Fig. 6b-h). Moreover, the effects of BMSC-Exos were in a dose-related manner so 50 μg/mL was selected for the following experiments.

To clarify whether BMSC-Exos promote angiogenesis of HUVECs by transferring specific miRs, the expression levels of Let-7i-5p, miR-21-5p, miR-22-3p and miR-486 in HUVECs were analyzed. Results showed that miR-21-5p expression was significantly increased in BMSC-Exos treated HUVECs (Fig. 7a), which was consist with the results in vivo. To further investigate the mechanisms, BMSCs were transfected with miR-21-5p inhibitor or NC. The miR-21-5p expression in BMSCs and BMSC-Exos both were downregulated as analyzed by qRT-PCR (Fig. 7b-c). Furthermore, the miR-21-5p expression in HUVECs administrated with miR-21-5p inhibitor transfected BMSC-Exos (miR-21-5p-Exos) was downregulated as well (Fig. 7d). Upon downregulation of miR-21-5p, the effects of BMSC-Exos on
HUVECs proliferation, migration and tube formation were eliminated (Fig. 8a-g). Thus, our results indicated that the proangiogenic activity of BMSC-Exos was partially mediated by miR-21-5p.

**BMSC-Exos upregulated VEGF and VEGFR2 expression in HUVECs by transferring miR-21-5p**

To examine the mechanisms of proangiogenic action of BMSC-Exos, the VEGF and VEGFR2 protein expression in HUVECs were detected by western blot analysis. Results showed that BMSC-Exos remarkably improved the expression levels of VEGF and VEGFR2. However, this effect was reversed by the miR-21-5p inhibitor (Fig. 8h-j). The above results revealed that BMSC-Exos enhances VEGF and VEGFR2 expression by transferring miR-21-5p into HUVECs.

**Discussion**

In the present study, we firstly confirmed that systemic treatment of BMSC-Exos could improve cerebral ischemia injury and promote angiogenesis in mice. Simultaneously, BMSC-Exos increased proangiogenic proteins expression, including VEGF, VEGFR, Ang-1 and Tie-2, and upregulated miR-21-5p expression in the ischemic boundary regions. Then, we transfected miR-21-5p inhibitor into BMSCs and further confirmed BMSC-Exos improved HUVECs angiogenesis via transferring miR-21-5p in vitro.

Increasing evidence suggests that BMSCs exert their therapeutic effects through paracrine mechanisms including secretion of Exos [35, 36]. Moreover, Exos not only show the same effects as BMSCs, but also show the advantages on targeted delivery, low immunogenicity, and high repairability [37]. BMSCs have been proven to secret Exos that show the potential for treatment of ischemic disease including ischemic stroke [38]. Recent studies showed that Exos derived from BMSCs promoted activation of microvascular endothelial cells and played an antiapoptotic role in cerebral ischemia/reperfusion injury [39]. In addition, BMSC-Exos could protect against myocardial ischemia reperfusion injury [40, 41]. In this work, we found that BMSC-Exos could promote angiogenesis and improve ischemia/reperfusion injury in mice. Moreover, we also confirmed that BMSC-Exos promoted the proliferation, migration, and tube formation of HUVECs in vitro. Thus, our research presents a new evidence in favor of the concept that BMSC-Exos possess a potent proangiogenic activity.

Nowadays, accumulated studies have shown that angiogenesis is essential for brain tissue repair following stroke, and the promotion of angiogenesis is broadly recognized as a promising therapeutic strategy [42, 43]. Angiogenesis is commonly defined as a multi-step process, involving proliferation, sprouting, migration and tube formation from the pre-existing vasculature [44]. It is regulated by multiple proangiogenic factors, among which VEGF, VEGFR2, Ang-1, and Tie-2 play the most important roles. The first step of angiogenesis is to remove the mural cells from the endothelial cells of established blood vessels, which results in an unstable blood vessel. Then, VEGF bonds to its receptor VEGFR2 and triggers downstream angiogenic signals, which lead to endothelial cells in these unstable blood vessel display different phenotypes: migratory tip cells or proliferating stalk cells [45, 46]. In the presence of VEGF and other angiogenic factors, endothelial cells proliferate and migrate and then form new vascular tube [47]. However, the newly formed endothelial cell tubes are unstable due to the lack of pericytes. Ang-1 is one of
the key angiogenic factors for the recruitment of the pericytes, and it act through the tyrosine kinase receptor Tie-2. The Ang-1/Tie-2 signal regulates maturation of newly formed vasculature, which eventually results in complex vascular network formation [48, 49]. In this study, we found that BMSC-Exos could dramatically improve the levels of VEGF, VEGFR2, Ang-1, and Tie-2 in the peripheral zone of ischemic mice. Unfortunately, we could only find the improved expression of VEGF and VEGFR2 in HUVECs after BMSC-Exos administration.

MiRs are a class of non-coding RNA that influence nearly all fundamental biological processes, and are stable in Exos due to the prevention of RNase degradation [50]. To date, a considerable amount studies have reported miRs related to angiogenesis. MiR-22 could regulate endothelial angiogenesis, inflammation, and tissue injury by targeting vascular endothelial-cadherin [51]. MiR-486-5p released by adipose-derived stem cell-derived EV could mediate wound healing and promote angiogenesis [52]. MiR-21-5p could promote extracellular matrix degradation and angiogenesis in the unilateral anterior crossbite model [53]. More importantly, it has been reported that miR-486-5p, miR-22, miR-21-5p and Let-7i-5p were abundant in BMSC-Exos [28]. Thus, we detected the expression of Let-7i-5p, miR-21-5p, miR-22-3p, and miR-486 in ischemic boundary region of stroke mice and HUVECs. The qRT-PCR data indicated that BMSC-Exos up-regulated the expression of these miRs, among which miR-21-5p was the most significant one. Then, we transfected miR-21-5p inhibitor into BMSCs, the expression of miR-21-5p in BMSC-Exos was significantly decreased confirmed by qRT-PCR. More importantly, the proangiogenic effects of BMSC-Exos on HUVECs were greatly abolished by miR-21-5p inhibitor. Moreover, the expression levels of VEGF and VEGFR2 in HUVECs were also significantly reduced by exosomes derived from BMSCs transfected with miR-21-5p inhibitor. These results suggest that the therapeutic effects of BMSC-Exos on angiogenesis may depend on the transport of miR-21-5p. However, although our study found that BMSC-derived exosomal miR-21-5p promoted angiogenesis in cerebral ischemia mice, the target genes of miR-21-5p and the detail mechanism need to be further investigated.

**Conclusions**

Our study indicates that BMSC-Exos can promote angiogenesis by transfer of miR-21-5p after stroke in mice. These findings show that BMSC-Exos might be a promising candidate for stroke treatment, and that BMSC-Exos could be tailored to maximize therapeutic effect.

**Declarations**

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Not applicable.

**Authors’ contributions**

H.H., X.W.H.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; L.L.: conception and design, provision of study material, collection and assembly of
data, manuscript writing; J.J.G., Y.Y.: administrative support, provision of study material, collection data; Y.F.: supervision, financial support, data analysis and interpretation; J.D.X: data analysis and interpretation, manuscript writing; L.S.C.: conception and design, financial support, provision of study material, manuscript writing, final approval of manuscript, supervision.

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**Availability of data and materials**

All data generated or analyzed during this study are available from the corresponding author upon reasonable request.

**Ethics approval and consent to participate**

All animal experimental procedures were approved by the Experimental Animal Ethics Committee of Zhejiang Chinese Medical University (reference number: ZSLL-2017-058), and performed in accordance with the guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health.

**Consent for publication**

The authors declare that they consent to publication.

**Competing interests**

The authors declare no potential conflicts of interest.

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**Abbreviations**

BMSCs: Bone marrow mesenchymal stem cell; BMSC-Exos: Bone marrow mesenchymal stem cell-derived exosomes; MCAO: Middle cerebral artery occlusion; TTC: 2,3,5-triphenyltetrazolium chloride; BrdU: 5-Bromo-2-deoxyuridine; vWF: von Willebrand factor; qRT-PCR: Quantitative reverse transcription-polymerase chain reaction; HUVECs: Human umbilical vein endothelial cells; FBS: Fetal bovine serum; tPA: Tissue-type plasminogen activator; PBS: Phosphate-buffered saline; NTA: nanoparticle tracking analysis; TEM: transmission electron microscope; DAPI: 4’,6-diamidino-2-phenylindole; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; OD: Optical density; VEGF: Vascular
endothelial growth factor; VEGFR2: Vascular endothelial growth factor receptor 2; Ang-1: Angiogenin-1; Tie-2: Tyrosine kinase receptor-2; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; HRP: Horseradish peroxidase; ECL: Enhanced chemiluminescence

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Figures
Figure 1

Characterization of BMSCs. a P0 BMSCs showed short bar shapes and P3 BMSCs adopted a uniformly spindle-shaped population. Scale bars=200 μm. b Flow cytometric analysis of cell surface markers indicated that BMSCs expressed CD29 and CD90, but not for CD34 and CD45. BMSCs bone marrow mesenchymal stem cells, P0 passage 0, P3 passage 3

Figure 2

Identification of BMSC-Exos. a Cup-shaped morphology of purified BMSC-Exos (indicated with arrows) observed by TEM. Scale bar = 500 nm. b The particle size and particle concentration of BMSC-Exos

| CD9  | CD63 | TSG101 | GAPDH |
|------|------|--------|-------|
| BMSCs | BMSC-Exos | Supernatants |       |

GAPDH
analyzed by NTA. c Western blots of exosomal membrane markers CD9, CD63 and TSG101. BMSC-Exos bone marrow mesenchymal stem cell-derived exosome, TEM transmission electron microscope, NTA nanoparticle tracking analysis

**Figure 3**

BMSC-Exos administration improved behavioral outcomes and reduced infarction volume in MCAO mice. a Neurological deficit scores. b Corner test. Values are presented as mean ±SD (n=12). **P<.01 compared with MCAO. c Representative TTC stained images of mice coronal brain sections at 3 and 14 days after MCAO. d Quantification of infract volume. Values are presented as mean ±SD (n=8). **P<.01 compared with MCAO. BMSC-Exos bone marrow mesenchymal stem cell-derived exosomes, MCAO middle cerebral artery occlusion, TTC 2,3,5-triphenyltetrazolium chloride
Figure 4

BMSC-Exos administration promoted angiogenesis in the ischemic brain. a BrdU/vWF double immunofluorescence staining. Red: vWF, green: BrdU, merge: BrdU/vWF. Scale bar=200 μm. b Quantification of d BrdU+/vWF+ microvascular endothelial cells. Values are presented as mean ±SD (n=6). **P<.01. c-d Representative western blot analysis and quantification of densitometries of western blot band. Values are presented as mean ±SD (n=3). **P<.01. vWF von Willebrand factor, BrdU 5-bromo-2-deoxyuridine, BMSC-Exos bone marrow mesenchymal stem cell-derived exosomes, MCAO middle cerebral
artery occlusion, VEGF vascular endothelial growth factor, VEGFR2 Vascular endothelial growth factor receptor 2, Ang-1 Angiogenin-1, Tie-2 Tyrosine kinase receptor-2, GAPDH Glyceraldehyde-3-phosphate dehydrogenase

Figure 5

Expression of miRs in ischemic brain as determined by qRT-PCR. a Let-7-5p. b miR-21-5p. c miR-22-3p. d miR-486. Values are presented as mean ± SD (n=3), *P<.05, **P<.01, ns not significance. BMSC-Exos bone marrow mesenchymal stem cell-derived exosomes, MCAO middle cerebral artery occlusion
BMSC-Exos promoted the proliferation, migration and tube formation of HUVECs. a BMSC-Exos uptake by HUVECs. Scale bar=50 μm. b Cell viability upon different concentration of BMSC-Exos. Values are presented as mean ± SD (n=6), *P<.05, **P<.01. c Representative images of scratch assay for migration of HUVECs. Scale bar=200 μm. d Representative images of transwell assay for migration of HUVECs. Scale bar=100 μm. e Representative images of tube formation of HUVECs. Scale bar=200 μm. f Relative

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Figure 6
measurement of migration distance. Values are presented as mean ± SD (n=3). *P<.05, **P<.01. g Number of migration cells in transwell assay. Values are presented as mean ± SD (n=3). *P<.05, **P<.01. h Total length of tube formed by HUVECs. Values are presented as mean ± SD (n=3), **P<0.01. DAPI 4’,6-diamidino-2-phenylindole, MTT 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide, OD optical density, BMSC-Exos bone marrow mesenchymal stem cell-derived exosomes

Figure 7

Expression of miRs in vitro as determined by qRT-PCR. a The expression levels of Let-7-5p, miR-21-5p, miR-22-3p and miR-486 in HUVECs cocultured with BMSC-Exos. Values are presented as mean ± SD (n=3). **P<.01, ns not significance. b The expression level of miR-21-5p in BMSCs after miR-21-5p inhibitor transfection. Values are presented as mean ± SD (n=3). **P<.01. c The expression level of miR-21-5p in BMSC-Exos after miR-21-5p inhibitor transfection. Values are presented as mean ± SD (n=3). **P<.01. d The expression level of miR-21-5p in HUVECs cocultured with miR-21-5p inhibited BMSC-Exos. Values are presented as mean ± SD (n=3). **P<.01. BMSCs bone marrow mesenchymal stem cells, BMSC-Exos bone marrow mesenchymal stem cell-derived exosome, miR-21-5p NC miR-21-5p inhibitor negative control, miR-NC-Exos miR-21-5p NC transfected BMSC-Exos, miR-21-5p-Exos miR-21-5p inhibitor transfected BMSC-Exos
BMSC-Exos promoted the proliferation, migration and tube formation of HUVECs through transferring miR-21-5p. a Cell viability of HUVECs cocultured with miR-21-5p- or miR-NC-Exos. Values are presented as mean ±SD (n=6), **P<.01. b Representative images of scratch assay for migration of HUVECs. Scale bar=200 μm. c Representative images of transwell assay for migration of HUVECs. Scale bar=100 μm. d Representative images of tube formation of HUVECs. Scale bar=200 μm. e Relative measurement of
migration distance. Values are presented as mean ±SD (n=3). **P<.01. f Number of migration cells in transwell assay. Values are presented as mean ±SD (n=3). **P<.01. g Total length of tube formed by HUVECs. Values are presented as mean ±SD (n=3). **P<.01. h-j Representative western blot analysis and quantification of densitometries of western blot band. Values are presented as mean ±SD (n=3). *P<.05, **P<.01. MTT 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide, OD optical density, BMSC-Exos bone marrow mesenchymal stem cell-derived exosomes, miR-NC-Exos miR-21-5p NC transfected BMSC-Exos, miR-21-5p-Exos miR-21-5p inhibitor transfected BMSC-Exos, VEGF vascular endothelial growth factor, VEGFR2 vascular endothelial growth factor receptor 2, GAPDH glyceraldehyde-3-phosphate dehydrogenase

**Supplementary Files**

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