Neural stem cell-derived exosomes transfer miR-124-3p into cells to inhibit glioma growth by targeting FLOT2

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Received February 20, 2022; Accepted June 8, 2022

DOI: 10.3892/ijo.2022.5405

Abstract. Currently, exosomes (EXOs) are being explored as novel drug delivery carriers with greater advantages, including crossing the blood-brain-barrier and loading drugs. The present study utilized EXOs derived from neural stem cells (NSCs) for the delivery of molecular drugs to treat gliomas. miR-124-3p was selected according to previous studies by the authors, and the effects of the delivery of miR-124-3p to glioma cells by NSC-EXOs in vitro and in vivo were evaluated. It was found that NSC-EXOs successfully delivered miR-124-3p into glioma cells, and NSC-EXOs loaded with miR-124-3p significantly inhibited glioma cell proliferation, invasion and migration. Furthermore, the delivery of miR-124-3p by NSC-EXOs suppressed flotillin 2 (FLOT2) expression by specifically binding to the 3’ untranslated region of the FLOT2 gene in gliomas; subsequently, AKT1 was found to be associated with the EXO-miR-124-3p/FLOT2 pathway. Moreover, the therapeutic effects of the delivery of miR-124-3p by NSC-EXOs were confirmed in a mouse tumor xenograft model of glioma. Thus, bio-carrier NSC-EXOs loaded with miR-124-3p suppressed glioma growth via the EXO-miR-124-3p/FLOT2/AKT1 pathway. On the whole, the present study provides insight into stem cell-free molecular-targeted therapy based on bio-carrier NSC-EXOs and provides a potential strategy for the treatment of glioma.

Introduction

Glioblastoma multiforme (GBM) is the most invasive and malignant tumor of the central nervous system (CNS) with a median survival rate of <14.6 months (1,2). Currently, in clinical practice, the common treatment strategy for patients with GBM is combined neurosurgical resection with chemotherapy or radiotherapy; however, the recurrence rate of GBM in patients remains very high. With the development of molecular-targeted therapies, there have been numerous pre-clinical studies on novel targeted drugs (3-5); however, an appropriate drug carrier has not yet been discovered in order to solve certain key issues, particularly the restrictive ability of the blood-brain-barrier (BBB) and drug maldistribution (4,5).

Neural stem cells (NSCs) are CNS stem cells and reside in two major neurogenic niches of the brain: the subventricular and subgranular zone. They have self-renewal properties and multipotential differentiation abilities (they can differentiate into neurons, astrocytes and oligodendrocytes) (6). Previous studies have demonstrated that NSCs can migrate to brain lesions, including tumors, which is closely associated with the cell paracrine mechanism (7-10). Exosomes (EXOs) secreted by cells are the main functional paracrine molecules (11,12). EXOs have a lipid bilayer structure with a diameter of 30-200 nm. They contain proteins, lipids, mRNAs and microRNAs (miRNAs/miRs), and are critically involve in intercellular communications through the transfer of exosomal cargo from the source cells to targeted cells. Moreover, EXOs have greater advantages, including the presence of active molecules from source cells, the ability to easily cross the BBB, immune privilege, the ability to deliver drugs and they can be used in targeted therapy (11-13). In addition, when EXOs are taken up by cells, such as tumor cells, they regulate the immune response, tumor occurrence and metastasis by transferring exosomal cargos (14-16). Of note, EXOs can be modified to carry small molecular drugs, including miRNAs, for individual-based targeted therapy.

miRNAs, as small non-coding RNAs, modulate gene expression at the post-transcription level by binding to ≥1 gene in their specific 3’-untranslated region (UTR). Furthermore, miRNAs are crucially involved in the occurrence and development of GBM (17). Previous studies, including a previous study by the authors, have demonstrated that miR-124-3p...
overexpression significantly inhibits GBM cell proliferation and migration, as well as angiogenesis and enhances glioma chemosensitivity (18-20). The small molecule miR-124-3p can be considered as a tumor suppressor gene in gliomas.

Thus, the present study aimed to use natural biological carrier EXOs derived from NSCs to load miR-124-3p as a molecular drug for GBM treatment, as well as to further explore the potential mechanisms underlying the regulation of GBM progression by EXOs carrying miR-124-3p.

Materials and methods

Glioma cells and NSC culture. The U87MG (glioblastoma of unknown origin) and U251MG (glioblastoma) cell lines were preserved in the authors' laboratory. U87MG cells were matched with the ATCC database by short tandem repeat (STR) analysis. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.). The medium was changed at 3-day intervals and the cells were passaged on the 6th or 7th day with dissociation using Accutase® (MilliporeSigma). The medium was changed at 2-day intervals and the cells were passaged on the 4th or 5th day through trypsinization (Trypsin; Gibco; Thermo Fisher Scientific, Inc.).

NSCs were generously provided by Professor Lukui Chen (Southern Medical University, Guangzhou, China); moreover, details regarding the cells have been previously described (21,22). NSCs were maintained in serum-free DMEM/F12 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 2% B27 (Gibco; Thermo Fisher Scientific, Inc.), 20 ng/ml basic fibroblast growth factor (PeproTech, Inc.), 20 ng/ml epidermal growth factor (PeproTech, Inc.) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.). The medium was changed at 2-day intervals and the cells were passaged on the 6th or 7th day with dissociation using Accutase® dissociation reagent (MilliporeSigma).

EXO isolation and characterization. EXOs were isolated from NSC culture supernatants using the Exo-spin™ Exosome Isolation and Purification kit (Cell Guidance Systems) as per the manufacturer's instructions. Briefly, conditioned medium (CM) was collected, centrifuged at 2,000 x g, 4˚C for 10 min and filtered to remove cell debris using a filter unit (MilliporeSigma) with a 0.22-µm membrane. As regards EXO isolation and purification, 1/2 volume of Exo-spin™ Buffer was added and mixed overnight, followed by centrifugation at 16,000 x g, 4˚C for 1 h. EXO pellets were resuspended in 100 µl cold PBS. The purified EXOs were then collected using the Exo-spin™ column through careful centrifugation at 50 x g, 4˚C for 1 min.

For transmission electron microscopy (TEM), a drop of EXOs was placed on a carbon-coated copper grid followed by the addition of a drop of 2% phosphotungstic acid to the stain for 5 min at room temperature. Subsequently, it was allowed to dry with a final examination using TEM (H-7650, Hitachi, Inc.). For nanoparticle tracking analysis (NTA), EXOs were loaded with miR-124-3p through electroporation using a Gene Pulser Xcell (Bio-Rad Laboratories, Inc.) electroporation system. First, miR-124-3p mimics (Guangzhou RiboBio Co., Ltd.) were mixed with EXOs in an electroporation buffer at a final concentration of 200 nM. The mixtures were then cultured at 4˚C for 10 min and transferred to ice-cold 0.2-cm cuvettes for electroporation at 400 V and 125 µF capacitance with a single pulse. Subsequently, the EXOs were maintained on ice for ≥15 min following electroporation. Negative control (NC) mimics (Guangzhou RiboBio Co., Ltd.) mixed with EXOs were electroporated to serve as a control.

Immunofluorescence. The NSCs (including neurospheres and mononaplasts) and their differentiated cells (neurons, astrocytes and oligodendrocytes) (cell differentiation was induced using differentiation medium as follows: DMEM/F12 supplemented with 1% B27, 2% FBS and 1% penicillin-streptomycin, cultured for 7-10 days) were washed and fixed using 4% paraformaldehyde at room temperature for 30 min. After washing and fixation, the cells were incubated with primary rabbit antibodies at 4˚C overnight. Following incubation with secondary antibodies [goat anti-rabbit 488 (1:600, cat. no. ab129819, Abcam), antibodies at 4˚C for overnight. Following incubation with secondary antibodies (goat anti-rabbit 488 (1:600, cat. no. 4412, Cell Signaling Technology, Inc.), and mouse anti-Musashi1 (1:200, cat. no. ab18207, Cell Signaling Technology, Inc.) and goat anti-mouse 555 (1:600, cat. no. 4409, Cell Signaling Technology, Inc.) at room temperature for 1 h, the slides were
mounted with DAPI (Beyotime Institute of Biotechnology), followed by examination under an inverted fluorescence microscope (IX73, Olympus Corporation) with light incubation being avoided.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from all samples (glioma cells) using the Total RNA kit (Omega Bio-Tek, Inc.) as per the manufacturer’s instructions. cDNA synthesis was performed using the Transcriptor First Strand cDNA Synthesis Kit (Invitrogen; Thermo Fisher Scientific, Inc.) and qPCR was performed using the ABI Detection System with FastStart Universal SYBR-Green Master (Roche Diagnostics) following the manufacturer’s instructions. The primers used were as follows: 
mir-124-3p forward, 5’-ACACTCCAGCTGGTAAAGGCA CGCGGTGA-3’ and reverse, 5’-CTCAACTGTGTTCTG GGAATCGCATTCAAGTGTGGCATT-3’; flotillin 2 (FLOT2) forward, 5’TGGCTGACTTACAGGGACC-3’ and reverse, 5’TCCAGCGCAATCTCCTTGTCC-3’; 5% milk at room temperature for 1 h and probed with primary rabbit anti-FLOT2 (1:1,000, cat. no. 66881-1-Ig.), anti-PI3K (1:1,000, cat. no. bs-0061R, BIOSS) at 4˚C for overnight. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit, 1:10,000, cat. no. 115-035-003; goat anti-mouse, 1:10,000, cat. no. 115-035-003; both from Jackson ImmunoResearch Laboratories, Inc.); subsequently, they were reacted with an enhanced ECL substrate (32106, Pierce; Thermo Fisher Scientific, Inc.), imaged using a Chemiluminescence imager (ChemiScope 6300, Clinx Science Instruments Co., Ltd.), and analyzed using ImageJ software (version 1.50i; National Institutes of Health).

Plasmid construction and dual-luciferase reporter assay. The target sequences of the FLOT2 wild-type (WT) 3’-UTR containing the predicted miR-124-3p binding sites and its mutant (MUT) 3’-UTR were synthesized. The fragments were sub-cloned into the pmirGLO dual-luciferase reporter vector (Promega Corporation) as per the manufacturer’s instructions. For the luciferase reporter assay, glioma cells were cultured in 24-well plates and transfected with a complex of 50 nM miR-124-3p or NC mimics (Ribobio, Inc.) and the WT or MUT luciferase reporter vectors (OBI Technology Corp., Ltd.) at 37°C for 48 h. Normalization was performed using the Renilla luciferase construct. Following 48 h of incubation at 37°C, the luciferase activity was measured using a dual-luciferase reporter system (EI960, Promega Corporation).

RNA interference and transfection. Duplex siRNA targeting human FLOT2 were purchased from GengPharma. Co., Ltd. Following a 24-h incubation at 37°C with an antibiotic-free medium, the glioma cells were transfected with anti-FLOT2 small interfering RNA (siFLOT2, 20 μM) using Lipofectamine 2000® Transfection Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 3 days prior to the start of the subsequent experiments. The FLOT2-siRNA sequences were as follows: siRNA-1 sense, 5’-GCGUGUUGGUGCUCCGACU-3’ and antisense, 5’-CGACACACCAAGGGCUAGAU-5’; siRNA-2 sense, 5’-GCAAUUCCUGGGUAAAGAU-3’ and antisense, 5’-CGUCAAGACCAUUCCUAU-5’; siRNA-3 sense, 5’-CCAAGAUGGACUCUAA-3’ and antisense, 5’-GGUGCUCAGCUGAAGAU-5’. For the experiment using the AKT1 agonist, Sc79 (S7863, Selleck Chemicals LLC.), Sc79 (10 μg/ml) was used to treat the cells for 3 days following transfection with siRNA FLOT2; total protein was then extracted for western blot analysis.

Cell Counting Kit-8 (CCK-8) assay for cell viability assessment. The glioma cells were seeded in 96-well plates for cell viability analysis using the CCK-8 assay. After 24 h, EXOs or small molecules were added to the co-culture for 1-4 days; subsequently, 10 μl CCK-8 (Nanjing KeyGen Biotech Co., Ltd.) was added into each pore. After 2-4 h of incubation at 37°C, the absorbance value (OD 450 nm) of each pore was colorimetrically determined (Multiskan MK3, Thermo Fisher Scientific, Inc.).

Transwell invasion assays. Briefly, as regards the Transwell invasion assay, the U87MG and U251MG cells were added into 24-well plates with an 8-mm-pore polycarbonate membrane coated with 20 mg of Matrigel (BD Biosciences). The 2x10⁴ cells were cultured in the upper chamber in serum-free medium; additionally, medium containing 10% FBS was added to the lower chamber. Following a 24-h incubation at room temperature, the invasive cells were fixed using 4% paraformaldehyde, stained using 0.1% crystal violet (Beyotime Institute of Biotechnology) at room temperature
for 30 min, and photographed using an Inverted Microscope (IX73, Olympus Corporation). Three independent experiments were performed.

**Wound healing assays.** Briefly, for the wound healing assays, the U87MG and U251MG cells were seeded in complete medium of 6-well plates until at least 80% confluency was reached. Subsequently, a wound was created through scraper manual scraping. After removing the floating cells by washing twice using serum-free medium, the cells were incubated at room temperature for 24 h. Migrated cells were observed using an inverted microscope (IX73, Olympus Corporation). The values obtained were expressed as the cell migration percentage.

**Glioma xenograft model.** A total of 12 4-week-old BALB/c male nude mice were purchased from the Guangdong Animal Center. The present study was reviewed and approved (permit no. A2020-015) by the Institutional Animal Care and Use Committee of The Second Affiliated Hospital of Guangzhou Medical University, and was performed according to the guidelines of the Committee on Animal Research and Ethics. All mice were kept in an aseptic environment with a constant humidity (40-60%) and temperature (24±2°C) with a 12-h light/dark cycle. The animals were also provided with free access to food and water, and the padding was replaced twice a week. To establish the glioma model, U87MG cells (1x10⁷ cells in 100 µl PBS for each mouse) were subcutaneously injected into the right axilla of nude mice for 10 days; subsequently, EXOs loaded with miR-124-3p (concentration 4x10⁵ particles in 20 µl PBS/each time; untreated cells 20 µl PBS were used as a control, twice per week, n=6 mice/group) were used for tumor treatment by intratumoral injection, and the anesthesia method used was gas isoflurane (30% vol/min), and photographed, and the tumor volume and weight were measured, and photographed, and the tumor volume and weight were measured. The mice were fixed in formalin and embedded in paraffin or snap-frozen in liquid nitrogen.

**Bioinformatics analysis.** Predicted miRNA targets or predicted genes were detected using the miRWalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/), TargetScan (http://www.targetscan.org), and miRTar-Base (https://mirtarbase.cuhk.edu.cn/miRTarBase/miRTarBase_2022/php/index.php) databases. Furthermore, the miR Cancer database (http://mircaner.ecu.edu/index.jsp) and GEPIA database (http://geopia.cancer-pku.cn/index.html) were used to evaluate the expression of FLOT2 and its association with the overall survival (Kaplan-Meier method with the log-rank test) of patients with GBM derived from the database.

**Statistical analysis.** Statistical analyses were performed using GraphPad 8.0 software (GraphPad Software Inc.). Data were analyzed using a two-tailed Student's t-test and are presented as the mean ± standard deviation (mean ± SD). The significance of the differences between different groups was examined using one-way ANOVA followed by the Tukey-Kramer post hoc test. Values of P<0.05 and P<0.01 were considered to indicate statistically significant and highly statistically significant differences, respectively.

**Results**

**Characterization of EXOs derived from NSCs.** NSCs have self-renewal properties. When they were cultured in complete medium, the NSCs gradually formed increasingly large neurospheres over time (Fig. 1A). As shown in Fig. 1B, the NSCs expressed the typical NESTINneurosphere marker, as well as the stem cell markers, SOX2 and Musashi1. Furthermore, NSCs are capable of multipotential differentiation; specifically, following induction in differentiation medium (DMEM/F12 supplemented with 1% B27, 2% FBS and 1% penicillin-streptomycin), they gradually differentiated into neurons, astrocytes and oligodendrocytes, which expressed TuJ1, GFAP and MOG, respectively, as revealed by immunofluorescence analysis (Fig. 1C).

To evaluate NSC-derived EXOs, secreted vesicles were extracted from the NSC medium through standard super-centrifugation and SEC methods. EXO characterization was performed using NTA, TEM and western blot analysis methods based on the minimal information from research on extracellular vesicles 2018 (24). NTA revealed that the vesicle diameter mainly ranged from 30 to 200 nm (Fig. 1D), TEM revealed that the EXOs had a concave-cup morphology (Fig. 1E). Western blot analysis revealed that the EXOs significantly expressed exosomal classical markers, including Alix, Hsp90 and Tsg101; however, they did not express the cell protein (Golgi marker), GM130 (Fig. 1F). Taken together, these findings represented features of NSC-secreted EXOs; accordingly, we termed them as NSC-EXOs.

**NSC-EXOs loaded with miR-124-3p suppress glioma cell proliferation, invasion and migration.** To assess NSC-EXO uptake by the glioma cells, NSC-EXOs were labeled using PKH-67 followed by co-incubation with the U87MG and U251MG cells, and loaded miR-124-3p mimics into NSC-EXOs through electroporation. Following a 24-h co-incubation, PKH67-labeled NSC-EXOs and carrier Cy5-miR were significantly detected in the cytoplasm and nucleus of the glioma cells (Fig. 2A). It was found that NSC-EXOs can freely enter glioma cells. Subsequently, following the addition of the EXOs loaded with miR-124-3p to glioma cells, RT-qPCR revealed a markedly higher miR-124-3p expression in the EXO-miR-124-3p mimics group than in the control and NC groups for both the U87MG and U251MG cells (Fig. 2B).

Subsequently, the effects of NSC-EXOs loaded with miR-124-3p on glioma cell proliferation, invasion and migration were evaluated using CCK-8, Transwell and wound healing assays. Compared with the control and NC groups, the EXO-miR-124-3p group exhibited a significantly decreased cell proliferation, particularly at 48 h following the addition of EXOs to glioma cells (Fig. 2C). Compared with the control and NC groups, the EXO-miR-124-3p group exhibited...
a significantly lower number of invaded and migrated cells in the Transwell and wound healing assays, respectively (Fig. 2D and E). These results indicated that the NSC-EXOs could successfully transfer miR-124-3p into glioma cells to suppress tumor cell proliferation, invasion and migration.

**NSC-EXO-miR-124-3p inhibits glioma growth by targeting FLOT2.** Three miRNA bioinformatics databases (TargetScan, miRTarBase and miRWalk) were used to predict the potential target genes of miR-124-3p; subsequently, six key candidate genes were selected (Fig. 3A). Subsequently, the NSC-EXOs loaded with miR-124-3p were incubated with the glioma cells for 48 h; moreover, RT-qPCR was used to determine the mRNA expression of the six candidate genes in the glioma cells. Compared with the control and NC groups, the EXO-miR-124-3p group exhibited a significantly lower FLOT2 expression (Fig. 3B). However, relative to the control/NC group, the expression of **SUB1** was inconsistent in the EXO-miR-124-3p group of U87 and U251 cells (higher in U87 and lower in U251 cells), **SMAD** expression was only significantly lower in the U251 cells, but not in the U87 cells; and **RHOG** expression was higher in the U251 cells, and lower in the U87 cells (vs. the control). Thus, FLOT2 protein expression was then examined. Similarly, compared with the control and NC groups, the EXO-miR-124-3p group exhibited a significantly lower FLOT2 protein expression (Fig. 3C).

Consequently, the present study further investigated whether FLOT2 was the direct miR-124-3p target in glioma cells. A luciferase reporter plasmid containing the functional 3'-UTR and mutant 3'-UTR site of FLOT2 (termed pMIR-REPORT Luciferase-FLOT2 3'-UTR WT and MUT) was constructed (Fig. 3D). Following co-transfection of FLOT2 WT or MUT plasmid with either miR-124-3p mimics or NC in glioma cells for 48 h, a significantly decreased luciferase activity by ~57% was observed in the group co-transfected with miR-124-3p mimics and luc-FLOT2 3'-UTR WT than in the other groups (Fig. 3D). These findings demonstrated that EXOs-miR-124-3p directly targeted the 3'-UTR of FLOT2 and mediated glioma cell progression mainly by regulating FLOT2 expression.

**NSC-EXOs loaded with miR-124-3p suppress glioma cell progression via the FLOT2/AKT pathway.** siRNA targeting FLOT2 (siFLOT2) was constructed to knockdown FLOT2 expression in glioma cells and siRNA-2 was screened as the most efficient siFLOT2 (Fig. 4A), with the findings confirming a marked decrease in both the mRNA and protein expression.
of FLOT2 in glioma cells (Fig. 4B and C). Subsequently, the effects of siFLOT2 on glioma cell proliferation, invasion and migration were evaluated. CCK-8, Transwell and wound healing assays confirmed that siFLOT2 significantly suppressed glioma cell proliferation, invasion and migration, respectively (Fig. 4D-F). Taken together, these findings suggested that NSC-EXOs loaded with miR-124-3p regulated glioma cell progression mainly through the direct suppression of FLOT2 expression.

A previous study by the authors demonstrated that miR-124-3p promoted glioma progression mainly via the PI3K/AKT pathway (20). As shown in Fig. 4G, PI3K/AKT protein expression was examined and it was found that compared with the control and NC groups, there was a decreased p-AKT1/AKT1 expression in the siFLOT2 group; however, PI3K expression was unaltered (data not shown). Subsequently, the AKT1 agonist, Sc79, was used to rescue AKT1 expression in the siFLOT2 group. Compared with the si-FLOT2 group, the combined treatment group exhibited a higher AKT1 expression (Fig. 4G). These findings suggested that EXO-miRNA-124-3p/FLOT2 mediated glioma cell progression via the AKT1 signaling pathway. Thus, NSC-EXOs loaded with miR-124-3p suppressed glioma cell progression mainly via the FLOT2/AKT1 pathway.

**NSC-EXOs loading with miR-124-3p inhibits tumor growth in vivo.** To explore the effects of NSC-EXOs loaded with miR-124-3p on glioma cell growth in vivo, glioma cells were initially injected into the subcutaneous tissue of nude mice after 10 days to allow tumor growth at an appropriate volume. Subsequently, NSC-EXOs loaded with miR-124-3p were used for treatment, with PBS as a control. Compared with the control group, treatment using NSC-EXOs-miR-124-3p markedly suppressed tumor growth (Fig. 5A). Additionally, compared with the control group, the tumor-bearing mice in the treatment group exhibited a significantly smaller tumor volume and
weight in (Fig. 5B and C). This demonstrated that NSC-EXOs loaded with miR-124-3p also had a marked inhibitory effect on glioma cells in vivo with no toxic side-effects observed in the mice. The present study further examined FLOT2 expression in tumor tissue using RT-qPCR and western blot analysis. The results revealed the significant inhibition of FLOT2 and AKT1 expression (Fig. 5D and E) in the group treated with the NSC-EXOs loaded with miR-124-3p. In addition, the miRCaner and GEPIA databases were used to evaluate gene expression, as well as the association between FLOT2 expression and the overall survival of patients with GBM (Fig. 5F and G). The expression of hsa-miR-124 was found to be decreased in gliomas, which was consistent with the findings from a previous study (20) by the authors. The expression of FLOT2 was significantly increased in the tissues of patients with GBM. Furthermore, the overall survival of patients with GBM with a high FLOT2 expression was significantly lower than that of patients with a low expression. There was a negative association between miR-124-3p and FLOT2 in gliomas. Thus, NSC-EXOs loading with miR-124-3p inhibited glioma growth in vivo, which was consistent with the results obtained in vitro; moreover, NSC-EXO-miR-124-3p suppressed glioma cell progression mainly via the FLOT2/AKT1 pathway.

**Discussion**

The present study mainly explored the ability of natural biological drug carrier EXOs to deliver small molecular miRNA into glioma cells, and found that NSC-EXO-miR-124-3p significantly inhibited glioma growth. This was achieved by specifically suppressing downstream FLOT2 expression and downregulating the AKT1 signal pathway in gliomas (Fig. 6). Currently, EXOs are being developed as diagnostic biomarkers for some diseases (25-27); however, the therapeutic advantages
Figure 4. NSC-EXOs loaded with miR-124-3p suppress glioma cell progression via the FLOT2/AKT pathway. (A) Western blot analysis was used to examine FLOT2 expression following siRNA knockdown. (B) The efficiency of si-FLOT2 knockdown was determined using reverse transcription-quantitative PCR. (C) FLOT2 protein expression in glioma cells was detected using western blot analysis. (D) The proliferative ability of glioma cells transfected with si-FLOT2 or NC was examined using CCK-8 assay; the x axis represents the days. (E) Cell invasion was determined using Transwell invasion assay in glioma cells transfected with si-FLOT2 (original magnification, x100). (F) Cell migration was detected using wound healing assay in glioma cells transfected with si-FLOT2 (original magnification, x100). *P<0.05, **P<0.01 and ***P<0.001, statistically significant differences between the NC and siFLOT2 group. NC, siRNA negative control; NSC, neural stem cell; EXOs, exosomes; FLOT2, flotillin 2.
of EXOs remain to be completely exploited. Recent studies have confirmed that EXOs can be developed as new drug carriers, and can be used to deliver small molecules, including miRNAs, to target cells, which has provided new insight into disease treatment, particularly for numerous refractory diseases, including tumors (28,29). Furthermore, studies have confirmed that EXOs can successfully transfer miRNAs to regulate the cell biological phenotype. For example, Wu et al (30) utilized bone marrow mesenchymal stem cell-derived exosomal miR-193a to reduce drug resistance in non-small cell lung cancer; Huang et al (31) demonstrated that EXOs from plasma of patients with medulloblastoma could transfer miR-130b-3p to inhibit medulloblastoma tumorigenesis; Munson et al (32) discovered that exosomal miR-16-5P was a target suppressor miRNA for malignant mesothelioma. Furthermore, the present study used bio-carrier NSC-EXOs for loading miR-124-3p and found that they could successfully deliver miR-124-3p into glioma cells to suppress tumor progression.

NSCs are exclusive brain stem cells involved in nerve tissue growth, development and repair. Moreover, NSCs are considered the most promising natural resource in the CNS and are capable of chemotaxis to lesions, including gliomas (7,8,10,33). The authors previously found that miR-124-3p expression was downregulated in glioma cells and tissues (20). Therefore, the present study used the advantages of NSCs and the capacities...
of carrier EXOs to load miR-124-3p into NSC-EXOs, named EXO-miR-124-3p. Subsequently, this molecular drug miRNA was successfully transferred into glioma cells to suppress tumor progression. The findings presented herein confirmed the hypothesis that carrier NSC-EXOs deliver miRNA-124-3p into glioma cells and are involved in inhibiting tumor cell proliferation, invasion and migration. Moreover, the effect of EXOs-miR-124-3p on glioma cell proliferation was about 20%, and the effects on cell invasion and migration were much more obvious, which was ~50%; therefore, EXO-miR-124-3p mediated the invasion and migration of glioma cells possibly via key downstream targets.

The present study then further investigated the potential mechanisms underlying the treatment effects of NSC-EXOs loaded with miR-124-3p on GBM progression. For this purpose, six potential target genes of miR-124-3p from three bioinformatics databases (TargetScan, miRTarBase and miRWalk) were selected, with FLOT2 being the key candidate. FLOT2 expression was significantly decreased by NSC-EXOs loaded with miR-124-3p and glioma cells. The FLOT2 gene is associated with numerous cancer types (34-36). FLOT2 is involved in the formation of caveolae or caveolae-like vesicles, has the functions of mediating cell adhesion, and mainly regulates the process of cell invasion and migration. Hazarika et al (34) revealed that the increased expression of FLOT2 was associated with melanoma progression. Wang et al (35) reported an association of a poor survival outcome of patients with breast cancer with a high FLOT2 expression level. Wang et al (36) also confirmed that FLOT2 promoted hepatocellular carcinoma by modulating the cell cycle and inducing epithelial-mesenchymal transition. Herein, when assessing whether FLOT2 was the direct miR-124-3p target in glioma cells, double luciferase reporter gene assay revealed that miR-124-3p directly targeted the 3'-UTR of FLOT2. The proliferation, invasion and migration of U87 and U251 glioma cells were significantly reduced by the knockdown of FLOT2 expression in tumor cells. Thus, bio-carrier NSC-EXOs loaded with miR-124-3p mediated glioma cell progression (particularly cell invasion and migration) mainly by inhibiting FLOT2.

Additionally, AKT, which is a classical signaling pathway, is involved in cancer progression (37,38). The present study found that NSC-EXOs loaded with miR-124-3p mimics downregulated FLOT2 expression and simultaneously inhibited p-AKT1 expression. This was reversed by the addition of the AKT1 agonist, Sc79. This suggested that NSC-EXOs loaded with miR-124-3p mimics suppressed glioma progression mainly by inhibiting FLOT2 and downregulating the AKT1 pathway. Notably, NSC-EXOs loaded with miR-124-3p significantly reduced glioma growth in mice; as PBS group was used as a control in vivo, the use of NSC-EXOs loaded with miR-124-3p demonstrated the inhibitory effects of these NSC-EXOs on tumor growth. Taken together, these findings confirmed that bio-carrier NSC-EXOs could be used to deliver molecular drug miR-124-3p into glioma cells, with subsequent EXO-miR-124-3p to target the downregulation of the FLOT2/AKT1 pathway. This eventually suppressed the proliferation, invasion and migration abilities of glioma.

However, the present study also has some limitations. These include the fact that the stimulatory effect of the BBB was not examined, multiple targets may be involved in in EXO-miR-124-3p treatment which need to be determined, and no in situ xenograft model of glioma was used. Furthermore, the contents in EXOs involved in the regulation of glioma cells need to be further investigated. The authors aim to further explore other applications of bio-carrier NSC-EXOs, including the key components of NSC-EXOs in the future.

In conclusion, GBM is the most malignant and invasive tumor in the CNS, with a very poor prognosis. The present study exploited the ability of new biological carrier NSC-EXOs to deliver miR-124-3p mimics into gliomas. It was found that EXO-miR-124-3p could specifically target the 3'-UTR of FLOT2 to inhibit downstream FLOT2 expression. Furthermore, these EXOs downregulated the AKT1 signaling pathway and suppressed glioma cell growth. The findings of the present study provide evidence of stem cell-free molecular targeted therapy based on bio-carrier EXOs, and these findings may aid in the development of novel therapeutic strategies for neurological diseases in the future.

Acknowledgements
Not applicable.

Funding
The present study was supported by the Natural Science Foundation of Guangdong Province (grant no. 2022A1515011328) and the China Postdoctoral Science Foundation (grant nos. 2019TQ0071 and 2020M672592).

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
Authors' contributions

GZ and YeW conceptualized the study. YoW, DC and CW were involved in the study methodology. CQ, YoW, YJ and GZ performed the experiments. CQ, YoW and YJ were involved in the writing of the original draft. GZ and YeW were involved in the writing, reviewing and editing of the manuscript, and supervised the study. GZ and YeW confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was reviewed and approved (permit no. A2020-015) by the Institutional Animal Care and Use Committee of The Second Affiliated Hospital of Guangzhou Medical University, and was performed according to the guidelines of the Committee on Animal Research and Ethics.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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