Bone marrow mesenchymal stem cell-derived exosome uptake and retrograde transport can occur at peripheral nerve endings

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**ABSTRACT**

We investigated the occurrence of mesenchymal stem cell (MSC)-derived exosome uptake and retrograde transport at peripheral nerve endings using bone marrow MSCs (bMSCs) transduced with recombinant CD63-green fluorescent protein (GFP) lentiviral plasmid. GFP was used to track the release of bMSC-derived exosomes and the uptake and transport at peripheral nerve terminals, the dorsal root ganglion (DRG), and the spinal cord. \textit{In vitro} cell culture and injection of a CD63-GFP exosome suspension into the right gastrocnemius muscle of an \textit{in vivo} rat model were also performed. Fluorescence microscopy of co-cultured CD63-GFP exosomes and SH-SY5Y or BV2 cell lines and primary cultured DRG cells in a separate experiment demonstrated exosome uptake into DRG neurons and glia. Moreover, we observed both retrograde axoplasmic transport and hematogenous transport of exosomes injected into rat models at the DRG and the ipsilateral side of the anterior horn of the spinal cord using fluorescence microscopy, immunohistochemistry, and Western blot analyses. In conclusion, we showed that exosome uptake at peripheral nerve endings and retrograde transport of exosomes to DRG neurons and spinal cord motor neurons in the anterior horn can occur. In addition, our findings propose a novel drug delivery approach for treating neuronal diseases.

**Introduction**

Peripheral neuropathy is characterized by weakness and pain usually in the feet and hands. Peripheral nerve injury is a major public health burden. The current treatment method of relieving symptoms is unsatisfactory and usually leads to poor functional recovery \cite{1}. Recent research has suggested that exosomes, which are nanoscale extracellular vesicles that can be released by almost any cell type and are capable of mediating intercellular communication \cite{2}, play a role in promoting neuronal regeneration \cite{3}. Exosomes can facilitate the exchange of biomolecules such as proteins and RNA between cells \cite{4,5}. Their uptake mechanism is thought to determine if endogenously transported substances contained within exosomes can initiate biological reactions \cite{4–6}. During endocytosis, the exosomal membrane fuses with the plasma membrane and the exosome unloads its contents into the recipient cell and subsequently fuses with the endosomal membrane to form a multivesicular body \cite{6,7}.

Mesenchymal stem cells (MSCs) are pluripotent cells that can be derived from the mesoderm, adipose tissue, bone marrow, and umbilical cord blood, and have been used in the treatment of central nerve ischemic injury, degenerative eye disease and peripheral nerve injury \cite{8}. The conditioned medium of cultured MSCs can stimulate MSC function in tissue repair. Some studies proposed that this function of MSCs is attributed to nutrient factors and cytokines that are secreted by MSCs into the growth medium \cite{9,10}. A study by Lai et al. \cite{11}, clarified that exosomes secreted by MSCs are the factors responsible for promoting tissue repair. Indeed, other studies confirmed the importance of stem cell-derived vesicles in promoting tissue regeneration \cite{7}, growth of neural processes and neuroprotection \cite{12}, and regulating neuroinflammation and neurodegenerative diseases \cite{3}.

Exosomes derived from MSCs are formed from the endocytosis of proteins, miRNA, mRNA, and other small molecules into MSVs \cite{13–16}. Endocytosis by MSCs leads to the formation of the early endosome, which evolves into the late endosome (also known as multivesicular bodies; MVBs). MVBs then transport molecules, such as growth factors and cytokines \cite{4,5,14}, designated for cellular export to the cell membrane. The fusion of MVBs and the cell membrane forms exosomes \cite{2,15}. Under transmission electron microscopy (TEM), exosomes appear as goblet or globular-shaped entities \cite{4,5,14}. Exosomes released from parenchymal cells of the central nervous system can be taken up by microglia and neurons \cite{2,17}. In addition, exosome transport can occur...
between dendritic spines of the same cell, across synapses, and to efferent nerve endings [2,17].

Since MSCs can produce a large amount of exosomes [4–6] and the bioactive substances contained within exosomes will not degrade during transport, MSC-derived exosomes are ideal for drug delivery [6]. Moreover, the use of microvesicles reduces the risk of dysfunction and transformation of transplanted stem cells. Thus, exosomes offer an efficient non-cellular therapeutic approach for tissue regeneration [4,6]. Furthermore, peripheral nerve endings were reported to uptake and transport liposomes [18], which suggests the possibility of exosomes being similarly taken up by nerve terminals. However, there are currently a few studies on the application of MSC-derived exosomes in the peripheral nerve system. Thus, we designed this study to determine whether exosome uptake and transport can also occur in the peripheral nervous system, thereby assessing the application of exosomes as targeted drug carriers in the treatment of neuropathies.

Methods

Cell culture and reagents

Bone marrow MSCs (bMSCs) were harvested from the femurs and tibias of 6–8-week old rats as previously described [19] and were then cultured in α-MEM containing 15% fetal bovine serum (FBS), glutaMAX, and penicillin-streptomycin (Gibco, Thermo Scientific, New York, USA). Dorsal root ganglion (DRG) cells were isolated from newborn rats by enzymatic digestion as previously described [20] and cultivated in phenol-red-free neurobasal medium (Gibco, Thermo Scientific, New York, USA) containing B-27 supplement (Gibco, Thermo Scientific, New York, USA), 7.5 pg/mL nerve growth factor (Abcam, Cambridge, USA), glutaMAX, and 1 g/mL cholesterol (Sigma-Aldrich, Missouri, USA). SH-SY5Y cells (ATCC Cell Bank, Manassas, VA, USA) were cultured in Dulbecco’s modified Eagles medium (DMEM)/F12 medium (SH30272.01, Hyclone, San Jose, CA, USA) supplemented with 15% FBS and penicillin-streptomycin (Gibco, Thermo Scientific, New York, USA). BV2 cells (ATCC Cell Bank, Manassas, VA, USA) were cultured in RPMI 1640 medium supplemented with 10% FBS and penicillin-streptomycin.

LV5-CD63 plasmid construction, lentivirus packaging, and transfection

The rat CD63 (exosome marker) gene was cloned into the lentivirus vector LV5 (carrying a GFP sequence; Addgene, Cambridge, USA) to construct the LV5-CD63 plasmid. Packaging and purification were performed as previously described [21]. Briefly, 293 T cells (ATCC Cell Bank, Manassas, VA, USA) were co-transfected with LV5-CD63 and lentiviral packaging vectors pMD2.G and pSPAX2 (Addgene, USA) using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Grand Island, New York, USA). The viruses were collected from the culture supernatant 48 h post-transfection and were concentrated by ultracentrifugation at 20,000 rpm for 2 h at 4 °C before resuspension in phosphate-buffered saline (PBS). Subsequently, the 293 T cells were transfected with serial dilutions of concentrated lentivirus. After 4 days, transfection was assessed as previously described [22] using fluorescence microscopy to confirm the presence of GFP-positive cells. Next, the bMSCs were transduced with the above-prepared lentivirus in 6-well plates at 37 °C overnight, and then cultured for 48 h in complete DMEM supplemented with puromycin to select GFP-positive bMSCs.

Quantitative real-time PCR

To detect CD63 expression in MSCs after lentiviral infection of the CD63-GFP construct, mRNA was extracted from CD63-GFP MSCs and negative control MSCs using Trizol reagent (Invitrogen, USA). Total RNA (500 ng) was reverse transcribed to cDNA using MMLV reverse transcriptase (Toyobo Co., Ltd., Osaka, Japan). Real-time PCR was performed using ABI PRISM 7500 (Thermo Fisher Scientific, USA) with Realtime PCR Master Mix containing SYBR Green (QPK-201, TOYOBO, Japan) and unique primers for CD63 and GAPDH. Primer sequences were as follows: CD63, Forward 5'-GGGAAGAGGAATGAA-3', Reverse 5'-CCATCCCCAAAGTTCACAG-3'; GAPDH, Forward 5'-ACAGCAACAGGGTGTTGGAC-3', Reverse 5'-TTTGAGGGTGCAACCAAGCT-3'.

Exosome isolation

bMSCs that reached 80% confluence (at passage 6) were washed with PBS three times and then incubated in serum-free medium for 3 days before harvesting. Exosome isolation was performed as previously described [23] with minor modifications. Briefly, the supernatant from bMSC serum-free cultures was collected by centrifugation at 300 g for 10 min at 4 °C to remove any intact cells. This was followed by centrifugation at 2000 × g for 20 min at 4 °C to remove dead cells and finally centrifugation at 10,000 × g for 30 min at 4 °C to remove cell debris. The supernatant was filtered through a 0.2 μm filter and then concentrated to about 9 ml using the Macrosep® Advance Centrifugal Device (MAP100C36, Pall, New York, USA) with a molecular weight cut-off of 100 kDa [24]. The concentrated sample containing exosomes was ultracentrifuged (Hitachi, Himac, CS150GX, Hokkaido, Japan) at 100,000 × g for 70 min at 4 °C. Exosome pellets were resuspended in PBS before storage at −80 °C.

Exosome preparation for uptake and co-culture experiments

bMSC-derived exosomes were ultracentrifuged at 100,000 × g for 70 min at 4 °C, washed with PBS and then ultracentrifuged again. The exosomes were then incubated with SH-SY5Y cells or BV2 cells for uptake experiments. For co-culture experiments, exosomes in 0.5 ml of bMSC (transfected with lentivirus LV5-CD63) conditioned media were incubated with DRG cells in 1.5 ml DRG culture media for 48 h. Cells were fixed and stained with either a GFAP (ab106509, Abcam,
Exosomes (5 and 0.5% laminin (Sigma-Aldrich, Missouri, USA) in media. Exosomes were prepared for TEM examination (Hitachi H7700, Tokyo, Japan) as previously described with minor modifications. Briefly, exosomes were incubated with SH-SYSY cells or BV2 cells (1 x 10^6) for 24 h at 37°C. Cells were fixed in 4% paraformaldehyde for 20 min at room temperature and washed with PBS. Uptake of labeled exosomes by SH-SYSY cells was visualized using confocal microscopy (Olympus FV1000, Tokyo, Japan). Z-stacks covering the entire cell volume were obtained and three independent experiments for each cell line were performed. Five visual fields were used to count labeled cells to calculate the mean transfection rate.

**Western blot analysis**

The exosome pellets were lysed in RIPA buffer (P0013B, Beyotime Biotechnology Co., Jiangsu, CHN) as previously described [25]. Lysates were separated on 10% polyacrylamide gels, transferred to a nitrocellulose membrane, and incubated overnight at 4°C with Alix (1:1,000; 2171 s, Cell Signaling, Boston, USA) and β-actin (1:5,000; A1978, Sigma-Aldrich, Missouri, USA) primary antibodies. Next, the blots were incubated with an HRP-conjugated rabbit anti-mouse IgG (1:5000; Thermo Fisher Scientific, New York, USA) secondary antibody. The bands were developed using an enhanced chemiluminescence reagent and the Tanon5500 imaging system (Tanon, Shanghai, CHN).

**Transmission electron microscopy (TEM)**

Exosomes were prepared for TEM examination (Hitachi H7700, Tokyo, Japan) as previously described with minor modifications. Briefly, exosomes in PBS were deposited onto a copper grid and then dried for 20 min at 40°C before staining with 1% phosphotungstic acid (Sigma-Aldrich, Missouri, USA) for 3 min. Excess staining solution was removed using a filter paper followed by air-drying for 20 min before visualization. Five random fields for each of the 10 samples were used to determine the average diameter of the exosomes.

**Flow cytometry and nanoscale flow cytometry**

bMSCs and exosomes were analyzed using flow cytometry (ACEA Biosciences Inc., NovoCyte 1040 Cytometer, San Diego, California, USA) and nanoscale flow cytometry (A50-Micro, Apogee Flow Systems, Hertfordshire, UK), respectively. bMSCs (passage 3) were first fixed in 2% paraformaldehyde for 15 min, washed twice with PBS and then labeled with rat CD45 FITC (1:1,000 dilution; 11–0461-80, eBioscience, Thermo Fisher Scientific, USA) or rat CD44 PE (1:400 dilution; 12–0444-80, eBioscience, Thermo Fisher Scientific, New York, USA) antibody for 1 h at 4°C in the dark before flow cytometric analysis. Data were analyzed using the NovoExpress™ software (ACEA Biosciences Inc., San Diego, California, USA). The exosome suspensions were diluted 100× before detection using the microflow method. The particle concentration was quantified by subtracting the PBS background concentration.

**Animal care and grouping**

Thirty-six 7–9 weeks old adult male SPF grade Sprague Dawley rats weighing 200–250 g (Changsha Tianqin Biotechnology Co. Ltd., Standard: GB14924.3 – 2010 Nutritional Composition of Mixed Feed for Laboratory Animals. Changsha, Hunan, CHN) were housed in standard rat cages at 21°C and 55% humidity under a 12:12 h light: dark cycle with unlimited access to water and food. All rats were cared for by trained personnel. Animal sacrifice and tissue collection were performed according to the guidelines of the Chinese Care and Use legislation and with the approval of the Animal Ethics Committee of Hainan Medical University. The rats were randomly divided into three groups (n = 12 per group): exosome injection group, exosome suspension injection after sciatic nerve transection group and PBS injection control group. For the exosome injection after sciatic nerve transection group, the gluteal muscles were separated to expose the right sciatic nerve, which was cut 1 cm distal and 5 mm away from the sciatic tubercle to prevent anastomosis. Penicillin was administered via an injection after suturing the muscles and skin.

**Gastrocnemius muscle injection**

Hundred-fold diluted exosome suspension or PBS (6 µl) was loaded into a micro-lift pump (10 µl Hamilton microinjector, GaoGe, Shanghai, CHN) and the right gastrocnemius muscle was injected at 0.1 µl per min over 60 min. The indwelling catheter was removed after an additional 5 min to prevent spillover. Animals in each group were euthanized by increasing the CO2 concentration and tissue fixation was performed for 2 h by perfusing 4% paraformaldehyde through the heart at days 1 and 5 after surgery. The ipsilateral lumbar (L)6 to the approximate sciatic (S)1 DRG and spinal cord were then harvested and frozen. Tissue sections were examined using fluorescence or confocal microscopy.

**Immunohistochemistry**

The DRG was subjected to stepwise alcohol gradient dehydration, paraffin infiltration at 60°C, and then sectioned (5 µm sections) and mounted on glass slides. Sections were deparaffinized with xylene followed by stepwise alcohol dehydration and heat-induced antigen retrieval, sealed in normal goat serum, and then incubated with CD63 (1:200 dilution; 108950, Abcam, Cambridge, USA) primary antibody at 4°C overnight. Subsequently, the sections were washed with PBS and incubated with PV-6001 (Beijing ZhongShan Golden Bridge Biotechnology Co., Beijing, CHN) secondary antibody at room temperature for 20 min. 3,3'-Diaminobenzidine (DAB)
was added to the sections and after the desired staining intensity was obtained, the reaction was stopped though stepwise alcohol gradient dehydration, xylene treatment, and finally sealing with neutral gum. One-hundred positively-stained cells in each group were randomly counted under the light microscope (Olympus FV1000, Tokyo, Japan) at 400 magnification and the gray value was calculated using the MetaMorph® NX microscopy (Molecular Devices, Sunnyvale, CA, USA) automation and image analysis software.

Fluorescence microscopy

DRG (10 nm thick) and spinal cord (15 nm thick) tissue blocks were encapsulated with OCT adhesive and sectioned at 20 °C after dehydration. Tissue was mounted on the microscope slide using the patch method, dried naturally, sealed with anti-fluorescence quenching tablet (S2100-25, Solarbio Science & Technology Co. Ltd., Beijing, CHN), and examined using fluorescence microscopy (Olympus FV1000, Tokyo, Japan). One-hundred positively-stained cells were randomly counted using the live cell imaging system at 400 magnification. The relative fluorescence intensity was analyzed using the MetaMorph® NX microscopy automation and image analysis software (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

Data from the immunohistochemistry experiment were analyzed using the SPSS version 19.0 software (IBM SPSS, Inc., Chicago, IL, USA). Data from six independent experiments are expressed as the mean ± standard error (mean ± SEM) calculated using Microsoft Excel software (Microsoft, Redmond, Washington, USA). A paired t-test was used to compare two groups. The mean value for the number of positively stained cells in the anterior horn of the spinal cord was calculated and the χ² test was performed. p Values of <.05 was deemed statistically significant.

Results

bMSC-derived exosomes were successfully transfected with CD63-GFP

The tetraspanin family of proteins [6], CD63, CD44, and CD9, are exosome markers. To prepare bMSCs carrying CD63-GFP-exosomes, the CD63-GFP recombinant plasmid was constructed (Figure 1(A)). Gel electrophoresis of the digested CD63-GFP recombinant plasmid showed that CD63 was approximately 1-kilobase (Figure 1(B)). Puromycin selection of bMSCs with LV5 expressing CD63-GFP revealed that infection efficiency was approximately 100% (Figure 1(C)). Fluorescence quantitative PCR showed that CD63 expression in the LV5-infected bMSC group was significantly higher compared to expression in the blank control group (Figure 1(D)). The LV5-CD63-GFP-infected bMSCs appeared green and multi-tuberculated (Figure 2(A)), and qRT-PCR showed that bMSCs transfected with CD63-GFP expressed CD63 of approximately 500 base pairs while empty bMSCs did not express CD63 (Figure 2(B)). CD44 expression, but not CD45 expression, was detected using flow cytometry (Figure 2(C)). Exosomes were isolated from the supernatant of cultured bMSCs using ultracentrifugation. The exosomes had diameters ranging between 30 and 150 nm as evidenced by TEM (Figure 2(D)). Microfluimetry of 100-fold diluted exosomes yielded an estimated concentration of 1.27 × 10^10/ml after subtracting the PBS background (2.92 × 10^9/ml), which accounted for 19.1% of the total supernatant concentration (1.56 × 10^10/ml). Western blot analysis showed that CD63 and Alix which is one of the proteins expressed on the surface of exosomes [16] were expressed in both un-transfected bMSCs and CD63-GFP-transfected bMSCs, but CD63 and Alix expression levels in the former were significantly higher (Figure 2(F)). Collectively, we showed that bMSCs can secrete exosomes and that CD63-GFP-exosomes can be used as a research tool.

In vivo uptake of bMSC-derived exosomes into nerve cells

After verifying the successful transfection of CD63-GFP in bMSCs, we investigated the uptake of CD63-GFP-labeled bMSC-derived exosomes into neurons and glial cells by coculturing exosomes with SH-SY5Y or BV2 neuroblastoma cells for 3–36 h using confocal laser scanning microscopy. After adding 2 μl of 100-fold diluted exosomes to each disc, we found that SH-SY5Y and BV2 cells had an uptake rate of 62.6% ± 5.8% and 83.8% ± 9.9%, respectively, at 36 h. Exosomes were also observed in the cytoplasm, pericytoplasm, and nuclei of these nerve cells (Figure 3(A,B)). Next, we measured the uptake of exosomes into primary cultured DRG glial cells and neurons. Exosome suspensions were added to cultured DRG cells within 12 h and growth saturation was reached at 36 h (Figure 3(C–E)). The rate of uptake (assessed by the number of GFP-positive cells) was 26.3 ± 6.0% in glial cells (167/529 cells) and 17.7 ± 6.7% in neurons (26/121 cells) at 36 h (Figure 3(E)).

Uptake of bMSC-derived exosomes into the DRG and anterior horn of the spinal cord

The uptake rate was 84.5% ± 8.50% (2310/2656 total cells) in DRG glial cells and 67.8% ± 7.09% (278/410 total cells) in neurons (Figure 4). Weak green fluorescence was observed in the bilateral spinal cord at day 5 after injection (Figure 4(D1)). We observed a significant increase in fluorescence intensity in the anterior horn of the spinal cord, with a higher number of positively-labeled neurons (15 vs. 5) and higher GFP intensity (80 vs. 50%) in the ipsilateral (injection side) compared to the contralateral side (Figure 4(E,F); p < .01) at day 5 after exosome suspension injection. We did not observe any green fluorescence at the spinal cord in the PBS injection control group.
Exosome uptake by nerve endings and retrograde axonal transport to the neuron body

To verify exosome uptake by nerve endings and retrograde axonal transport, we injected bMSC-derived CD63-GFP-labeled exosomes into the right sciatic nerves of the right gastrocnemius muscle (ipsilateral) of the rats. GFP-positive neurons and glial cells were observed (weak fluorescence signal) on both sides of the DRG 24 h after injection (Figure 5). At day 5 after injection, we observed a significant increase in the relative fluorescence intensity at the ipsilateral DRG of the control group (84.52 ± 9.60%; Figure 5(E); \( p < .01 \)). The GFP intensity for the sciatic nerve transected group was similar to that of the contralateral group (40.5 ± 6.54%). Negligible DRG fluorescence intensity was observed in the PBS injection control group. Immunohistochemistry (Figure 6(A–D) and Western blot analysis (Figure 6(E)) confirmed the fluorescence microscopy results, wherein CD63 expression was highest in the ipsilateral DRG of the control group at day 5 after injection (\( p < .01 \)).

Discussion

Poor patient outcome of peripheral nerve injury treatment approaches is a major clinical problem [1,8] and initiating neuronal regeneration is thought to be a promising therapeutic strategy. Exosomes carrying specific proteins or miRNAs have been shown to target neurons and promote their regeneration [4,5,14]. However, exosome uptake by
nerve endings and exosome transfer to the cell body has not yet been verified [1,3,5]. In this study, GFP labeling of the MSC exosome marker CD63 was used to track uptake and transfer of bMSC-derived exosomes [26]. CD63, also known as lysosomal granule membrane glycoprotein, is an important marker of platelet activation that is expressed on the surface of many cells. We showed that CD63-GFP-labeled exosomes secreted by bMSCs can be transferred into neurons and glial cells via the bloodstream, taken up by peripheral nerve endings and transported to DRG neurons and motor neurons in the anterior horn of the spinal cord. Thus, our findings propose using exosomes as vehicles for drug delivery in the treatment of neuronal diseases.

Figure 2. Characterization of exosomes. (A) Puromycin selection of bMSCs expressing CD63-GFP plasmid. Scale bar = 5 μm. (B) Agarose gel electrophoresis. (C) Flow cytometry profiles of paired marker proteins CD44 and CD45. (D) Electron micrograph (EM) profiles showing exosomes in the MSC culture supernatant (left) and control (middle), and a schematic diagram of the distribution of the average diameter of exosomes observed under EM (right). (E) Flow cytometry profiles. (F) Western blot image showing CD63 and Alix expression. CD63-MSC: CD63-MSC cell lysate; CD63-EXO: CD63-MSC exosomes; NC-EXO: NC-MSC exosome (negative control).
Lipophilic fluorescent dyes PKH67 and PKH26 or lipophilic fluorophore Dil and dio-labeled exosomes can be used to track the movement of exosomes in vitro and intracellular studies. However, dye residues may not be thoroughly removed by ultracentrifugation, and the transfection of dye residue or labeled exosomes cannot be distinguished. Thus, we used the lentiviral method to transduce CD63-GFP into rat bMSCs in this study and demonstrated that the extracted and purified exosomes stably expressed CD63-GFP, through microflow detection and western blot analysis.

Hematopoietic cell surface markers, such as CD44, are known to be expressed at low levels in bMSCs [27]. We identified bMSCs using flow cytometric detection of paired hematopoietic cell surface marker CD44 and non-marker CD45 (95.41% CD44+, CD45−). Our findings indicate that we were successful in culturing bMSCs with high purity.

There is a lack of standardized methods to separate, quantify, and characterize exosomes [27,28]. Common methods for exosome separation include ultracentrifugation, polymer precipitation, membrane affinity, antibody affinity precipitation, and gel exclusion [27–33], with ultracentrifugation being the gold standard for exosome separation due to its relatively high purity [15]. Sucrose gradient centrifugation is also commonly used to purify exosomes. Exosomes can be identified using Western blot analysis and electron microscopy, the latter of which has been the gold standard. Quantifying exosomes is dependent on their physical characteristics, such as size, mass, and density, or their surface proteins. Commonly

Figure 3. Exosome uptake by SH-SY5Y, BV2, and primary cultured DRG cells in vitro. Confocal laser scanning microscopy images of exosomes (green) derived from bMSCs co-cultured with (A) SH-SY5Y cells or (B) BV2 cells for 24 h, and with DRG cells for 3–36 h. Blue arrows indicate exosomes observed outside the cell membrane; white arrows denote exosomes observed around the cell membrane; and red arrows indicate exosomes that have entered cells. Exosomes were observed in the cytoplasm of (C) glial cells and (D) neurons. (E) Profile showing the change in the transfection rate in DRG neurons and glial cells with culture time. n = 6; Scale bar = 20 μm.
Figure 4. Exosome uptake by DRG cells and motor neurons of the anterior horn of the spinal cord at L5 and L6 segments in vivo. (A1) Open field photo of the DRG; (A2) DAPI staining of the DRG; and (A3) GFP-labeled DRG cells (green); Scale bar = 20 μm. (B) Merged image of A1, A2, and A3. Exosomes surrounding the nuclei of neurons are indicated with the arrowhead; exosomes inside the nuclei of neurons are indicated with the arrow. The bigger box is a zoomed-in image of the smaller box. (C) Profile showing the rate of exosome uptake by DRG glial cells and neurons in vivo. (D) Fluorescence micrographs showing GFP-positive neurons in the bilateral anterior horn of the spinal cord at the L5 and L6 segments on (D1) day 1 and (D2) day 5 after exosome injection. (D3) No GFP-positive neurons were observed in the PBS injection control group. Scale bar = 400 μm. (E) Profile showing the number and the (F) relative fluorescence intensity of neurons containing exosomes at the ipsilateral and contralateral sides of the anterior horn of the spinal cord on day 5 after exosome injection. n = 6. The asterisk indicates comparison with the opposite side, p < 0.01.
Figure 5. Exosome uptake by peripheral nerve endings and retrograde transport to DRG cells in vivo. Fluorescence micrographs (GFP, left; DAPI, middle; merged, right) showing the (A) ipsilateral side (injection side) of the DRG in a normal rat; (C) DRG section after the ipsilateral sciatic nerve was transectioned prior to exosome injection; and (B, D) contralateral side of the DRG in (B) normal and in a (D) rat where the right sciatic nerve was transectioned, respectively. Scale bar = 20 μm. (E) Profile showing the relative fluorescence intensities of the bilateral DRG in the PBS injection control group (PBS), at day 1 in the exosome injection group (EXO-1d), day 5 in the exosome injection group (EXO-5d), and day 5 in the injection in the ipsilateral sciatic nerve transectioned group (Injured-EXO-5d).
used quantitative techniques include immunoaffinity capture (IAC), surface plasmon resonance (SPR) [30], nanoparticle tracking analysis (NTA) [31,32], dynamic light transparency (DLS), paintbrush flow field fractionation coupled with multi-detection (AF4) and multiple detection coupling technology [33]. In this study, we used the ultracentrifugation method to isolate exosomes and confirmed exosome isolation as membranous vesicle-like particles using TEM. Quantification of

Figure 6. Immunohistochemical and Western blot analysis of CD63 in DRGs after exosome suspension injection. Immunohistochemical images showing the contralateral and ipsilateral sides of the DRG on day 5 (A) before (intact EXO) and (B) after the ipsilateral sciatic nerve was transectioned prior to exosome injection (injured EXO); and (C) the DRG of the PBS injection control group (intact PBS) on day 5 after exosome injection to the right gastrocnemius muscle. (D) Semi-quantitative immunohistochemical profile of the relative gray values of CD63 expression in the intact PBS, intact EXO, and injured EXO groups. n = 6. (E) Western blot image showing the relative CD63 protein expression in the DRGs in the five groups: intact EXO contralateral (con) and ipsilateral (ips) groups; injured EXO con and ips groups; and the intact PBS control group. n = 6. The asterisk indicates comparison with the opposite side and other groups, p < 0.01.
exosomes was performed using microflow detection and Western blot analysis.

Overnight incubation showed that cells were capable of exosome uptake [34]. We observed similar uptake when co-culturing exosomes with either SH-SY5Y or BV2 cells. SH-SY5Y cells can undergo rapid growth, reproduce at speeds similar to cancer cells, and display similar morphological, physiological, and biochemical functions of nerve cells. Hence, SH-SY5Y cells are typically used in place of primary DRG neurons, which are difficult to culture [35,36]. Similarly, BV2 cells, which are derived from mouse microglia, are used in place of primary DRG glial cells [37]. Hence, we co-cultured bMSC-derived exosomes with these two cell lines and indirectly demonstrated the uptake of exosomes by DRG neurons and glial cells. We established the in vitro primary DRG culture model to confirm the results obtained for the SH-SY5Y and BV2 cell lines but observed lower uptake rates indicating the weak viability of the primary DRG culture.

In this study, we injected exosomes into the gastrocnemius muscle because the long distance from the nerve endings to the cell body allowed us to exclude any effects of local dispersion upon detecting exosomes in the spinal motor neurons and/or DRG sensory neurons. Thus, we could conclude that there was successful retrograde axoplasmic transport and hematogenous transport of exosomes. To exclude the influence of transport via the bloodstream, we designed two test groups: the normal right gastrocnemius injection group and the transected right sciatic nerve followed by ipsilateral gastrocnemius injection group. Detection of GFP-positive cells in both sides of the DRG at day 1 after injection suggests the uptake of exosomes through hematogenous transport. The significantly stronger GFP intensity at the DRG and the ipsilateral (right side) of the spinal cord at day 5 after injection suggests that exogenous exosomes may have undergone retrograde axoplasmic transport. Initially, we examined the bilateral DRG on days 2 and 3 after gastrocnemius muscle injection but observed a much smaller increase in fluorescence intensity compared to day 5 after injection, indicating that axoplasmic transport is slow. Hence, we opted to use a 5-day model and made a deeper incision of the ipsilateral gastrocnemius muscle because the long distance from the nerve ending to the cell body could have prevented axoplasmic transport of exosomes in the spinal motor neurons.

Uptake of the exogenous exosomes in the spinal cord was similar to that of the bilateral DRG, except that GFP-positive cells could not be detected in the spinal cord on day 1 after exosome injection, which suggests that transport via the bloodstream requires a relatively longer time. This longer transport duration may be due to transport across the blood–nerve barrier. The ability of nanoparticles to transfer their substances and initiate biological reactions is determined by the mechanism by which they are absorbed [4–6]. Endocytosis is generally divided into phagocytosis and pinocytosis, wherein the former is usually employed by specialized phagocytes [38] and the latter is used by all cells. Pinocytosis is further divided into clathrin-dependent endocytosis (CDE), clathrin-independent endocytosis, and macropinocytosis (MP). The uptake of fluorescently-labeled extracellular vesicles was found to be mediated via CDE and MP [39].

Motor neurons are particularly important for peripheral nerve injury recovery and repair [40]. We showed that bMSC-derived exogenous exosomes can reach the motor neurons in the anterior horn of the spinal cord following gastrocnemius muscle injection via the blood circulation and by retrograde axoplasmic transport to the neuron cell body. In the peripheral nervous system, the basic repair mechanism of peripheral nerve injury involves survival and axonal burst and extension of neurons when connecting neurons to target cells [16,39]. Hence, using exosomes as natural carriers of specific drugs holds promise in the treatment of peripheral nerve diseases, especially in peripheral nerve regeneration therapy.

In conclusion, our findings revealed that the uptake of MSC-derived exosomes by peripheral nerve terminals in vivo is possible and that exosomes can undergo retrograde axoplasmic transport to neuronal cells, which provides new insight for the application of exosomes as targeted drug carriers to treat peripheral nerve diseases.

Disclosure statement
No potential conflict of interest was reported by the authors.

Data availability statement
The datasets generated and analyzed during the present study are available from the corresponding author upon reasonable request.

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