

ORIGINAL ARTICLE

Exosome-Based Delivery of miR-124 in a Huntington's Disease Model

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ABSTRACT

Objective Huntington's disease (HD) is a genetic neurodegenerative disease that is caused by abnormal CAG expansion. Altered microRNA (miRNA) expression also causes abnormal gene regulation in this neurodegenerative disease. The delivery of abnormally downregulated miRNAs might restore normal gene regulation and have a therapeutic effect.

Methods We developed an exosome-based delivery method to treat this neurodegenerative disease. miR-124, one of the key miRNAs that is repressed in HD, was stably overexpressed in a stable cell line. Exosomes were then harvested from these cells using an optimized protocol. The exosomes (Exo-124) exhibited a high level of miR-124 expression and were taken up by recipient cells.

Results When Exo-124 was injected into the striatum of R6/2 transgenic HD mice, expression of the target gene, RE1-Silencing Transcription Factor, was reduced. However, Exo-124 treatment did not produce significant behavioral improvement.

Conclusion This study serves as a proof of concept for exosome-based delivery of miRNA in neurodegenerative diseases.

Key Words Huntington's disease; microRNA; R6/2; miR-124; exosome.

Huntington's disease (HD) is a genetic neurodegenerative disease that is caused by the abnormal expansion of CAG repeats in the gene encoding huntingtin. HD results in devastating neurological symptoms, including cognitive impairment, neuropsychiatric symptoms, and involuntary choreiform movements.¹ Although the precise mechanisms of HD neurodegeneration remain unclear, one of the mechanisms involves alterations of transcriptional regulators, such as RE1-Silencing Transcription Factor (REST).² Mutant huntingtin no longer silences the activity of REST, and this loss results in increased binding of REST to RE1/neuron restrictive silencer element, producing transcrip-

tional dysfunction.³ Thus, many therapeutic approaches have attempted to restore normal REST expression.⁴

MicroRNAs (miRNAs) are short, non-coding RNAs of ~22 nucleotides that regulate gene expression by suppressing the translation of mRNA. We and others have previously shown that the expression of miRNAs is altered in HD⁵ and that miR-124 is one of the key miRNAs that is repressed in HD.^{6,7} In HD, decreased expression of miR-124 increases the level of its target gene, REST, resulting in the repression of key target genes such as brain-derived neurotrophic factor.⁸ miR-124 induces adult neurogenesis in the subventricular zone⁹ and regulates the cell cycle

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in striatal neurons.¹⁰ Given that the HD striatum exhibits decreased neurogenesis, which leads to brain atrophy,^{11,12} the delivery of miR-124 may be a feasible way to induce neural regeneration. However, naked miRNAs are vulnerable to degradation, and our recent experiments using naïve miR-124 in HD showed little therapeutic effects.¹³

Exosomes are endocytic nano-vesicles (< 100 nm) that are released by cells and that can deliver miRNA to recipient cells.¹⁴ Recently, because of their significance in normal physiology and disease progression, exosomes have been actively studied as a type of therapeutic agent¹⁵⁻¹⁷ and drug delivery vehicle.¹⁸ Exosomes can be isolated from various tissues,¹⁹⁻²² and many cells in the central nervous system also release exosomes, which participate in the intercellular transfer of DNA, RNA, or misfolded proteins; as a result, exosomes have been implicated in development, normal function, and pathogenesis.²³ Considering the importance of miRNA and exosomes in neurodegenerative diseases, exosomes containing miRNAs that are abnormally altered in neurodegenerative diseases should be studied to characterize their effects on disease models. Here, we investigated the feasibility of exosome-mediated therapeutic delivery of miRNA in a HD model.

MATERIALS & METHODS

Generation of miR-124-expressing cell line

Mature miR-124 was cloned into the pSUPER plasmid to overexpress the miRNA in cells. HEK 293 cells were transfected with the pSUPER-miR-124 vector using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The pSUPER-miR-124-transfected cells were cultured in medium containing Zeocin (500 µg/mL) to select for cells expressing miR-124. The medium was changed every 2 or 3 days until single-cell colonies formed (2–3 weeks). Single colonies were isolated and regrown for 2 weeks with Zeocin media, and miR-124 expression was tested using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Isolation of exosomes

HEK 293 cells overexpressing miR-124 were cultured in exosome-free Dulbecco's modified Eagle's medium (DMEM; Gibco Invitrogen/Life Technolo-

gies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco Invitrogen/Life Technologies) and 1% penicillin-streptomycin at 37°C in a 5% CO₂ incubator. To deplete bovine exosomes, DMEM containing 10% FBS was centrifuged at 100,000 g for 15 h, and the supernatant was used for cell culture. Culture supernatants were harvested after 5 days. Cells and cellular debris were eliminated from the conditioned medium by centrifugation at 3,000 g for 10 min at room temperature. The conditioned medium was treated with citrate (3 mM, 6 mM) for 2 days at 37°C. Exosomes were isolated using the Exo-Quick exosome precipitation kit (System Biosciences, Mountain View, CA, USA) according to the manufacturer's specifications. Briefly, 5 mL of conditioned medium was thoroughly mixed with 1 mL of Exo-Quick exosome precipitation solution, and the solution was incubated for 24 h at 4°C. The conditioned medium was centrifuged at 1,500 g for 30 min, and then the supernatant was removed and centrifuged at 1,500 g for 5 min. The exosome pellet was resuspended in buffer.

Analysis of intercellular transfer of Exo-124

Exosomes were labeled with PKH67 (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. Briefly, the exosome pellet was suspended in 100 µL PBS and then diluted in 1 mL of Diluent C. The solution was then rapidly mixed with a freshly prepared solution of 4 µL of PKH67 dye in 1 mL of Diluent C. The mixture was incubated for 5 min. Labeling was stopped by the addition of 2 mL of 1% BSA in PBS for 1 min. The samples were washed 2 times with 25 mL of PBS and then centrifuged at 120,000 g for 100 min. After labeling, the PKH67-labeled exosomes were stored in PBS containing 2% paraformaldehyde until flow cytometric analysis (BD Biosciences Pharmingen, San Jose, CA, USA). PKH67 fluorescence was evaluated using the green channel, and the data were analyzed using WinMDI software (Windows Multiple Document Interface for Flow Cytometry, West Lafayette, IN, USA). Fluorescently stained sections of brains that were injected with PKH67-labeled exosomes were analyzed using an inverted microscope (BX61, Olympus Corporation, Tokyo, Japan).

Real-time PCR for miRNA

RNA was isolated using Trizol (Thermo Fisher

Scientific, Asheville, NC, USA), and the level of miR-124 was quantified using the *mirVana* qRT-PCR miRNA Detection Kit and TaqMan miRNA assays (Ambion, Applied Biosystems, Foster City, CA, USA). All reactions were run in triplicate on an ABI PRISM 7,000 sequence detection system (Applied Biosystems). Relative expression levels were calculated using the comparative threshold cycle (Ct) method (relative expression levels = $2^{-\Delta Ct}$) and were normalized to the expression of the control, snoRNA202, which was measured in the same samples using an endogenous snoRNA detection kit (Ambion, Applied Biosystems).

HD transgenic mice and injection of exosome

The animal study was approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital, which was accredited by the International Association for the Assessment and Accreditation of Laboratory Animal Care. We used the R6/2 line of transgenic HD mice [B6CBA-Tg(HDexon1)62Gpb/3J, The Jackson Laboratory; 6 female and 6 male mice; 111 CAG repeats] and their wild type (WT) littermates (6 female and 6 male mice) at 6 weeks of age. These mice were obtained by crossing ovarian transplant hemizygous females with B6CBAF1/J males. Genotyping was performed using PCR.²⁴ An expanded CAG repeat in exon 1 of the HD gene is sufficient to cause a progressive neurological phenotype in these transgenic mice.²⁴ The mice were housed in groups with ad libitum access to food and water and a 12-h light/12-h dark cycle.

To inject exosomes, 6-week-old (42 days) mice were anesthetized via intraperitoneal injection of 1% ketamine (30 mg/kg) and xylazine hydrochloride (4 mg/kg) and then positioned in a stereotaxic apparatus. All exosomes obtained from 5 mL of conditioned medium were suspended in 12 μ L of PBS. In the exosome injection group (Exo-124), a 30-gauge Hamilton syringe was used to inject the exosome suspension into the bilateral striatum (2 μ L of suspension per hemisphere) at the following coordinates: AP + 0.38 mm, ML \pm 2.0 mm, and DV -3.5 mm from Bregma. The exosomes were slowly injected over a period of 5 min. The needle was left in position for another 5 min and then gently removed. All animal experiments and procedures complied with the NIH Guide for the Care and Use of Labora-

tory Animals.

Behavioral test

Rota-Rod performance was assessed using an accelerating Rota-Rod (San Diego Instruments, San Diego, CA, USA) as previously described.²⁵ The speed of the Rota-Rod was set to linearly increase from 4 to 40 rpm over 3 min. The mice were initially trained on the Rota-Rod for three consecutive days. Three trials were conducted per day, with a 30-min rest period between trials. The trials and tests were terminated if the animal fell off the rungs or gripped the device and completed two consecutive revolutions. At each age, mice underwent three trials, and the mean latency to fall was calculated.

Histological analysis

When the mice were 8 weeks old (1 week after the exosome injection, $n = 3$), histological evaluations were performed as previously described.²⁵ Briefly, the animals were deeply anesthetized and then perfused through the heart with 10 mL of cold saline and 10 mL of 4% paraformaldehyde in 0.1 M PBS. Sections (20 μ m thick) were counter-stained with DAPI (Sigma-Aldrich, St. Louis, MO, USA), and PKH67 fluorescence was analyzed using a microscopic imaging system (Carl Zeiss, Thornwood, NY, USA).

Western blotting and real-time PCR in R6/2 mice

At the age of 8 weeks (1 week after the exosome injection), the mice were sacrificed by decapitation, and the brains were immediately removed. Homogenates of each hemisphere were separately and serially processed for Western blotting and RNA work. Western blotting was performed using antibodies against REST (Abcam, Cambridge, UK), doublecortin (DCX; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or β -actin (Santa Cruz Biotechnology). The blots were developed using enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA) and digitally scanned (GS-700; Bio-Rad, Hercules, CA, USA). The optical density of each band relative to that of the β -actin band was determined using Molecular Analyst[®] software (Bio-Rad). For real-time PCR, total RNA was isolated from each hemisphere using QIAshredder and RNeasy kits (Qiagen, Valencia, CA, USA). miR-124 levels were measured using the *mirVana* qRT-PCR miRNA Detection Kit and TaqMan miR-

NA assays (Ambion, Applied Biosystems).

Data analysis and statistics

All data in this study are presented as the mean \pm standard deviation. The Mann-Whitney U test was used for nonparametric, inter-group comparisons. SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. A two-tailed p -value < 0.05 was considered significant.

RESULTS

We first generated a miR-124-expressing cell line

by transfecting the miR-124 expression vector into HEK 293 cells. miR-124 expression was confirmed by real-time PCR (data not shown). We harvested exosomes from the medium used to culture these cells, as presented in Figure 1A. Based on previous reports,²⁶ we added citrate and Mg^{2+} during the procedure to protect the RNA contained in the exosomes. Figure 1B shows the harvested exosome pellets.

We evaluated the pellets by blotting for CD9 and CD63, which are tetraspanin proteins that are expressed at the cell surface and thus serve as exosome markers.^{14,27} The exosomes expressed both CD9 and CD63 (Figure 1C), whereas centrifugation of unused

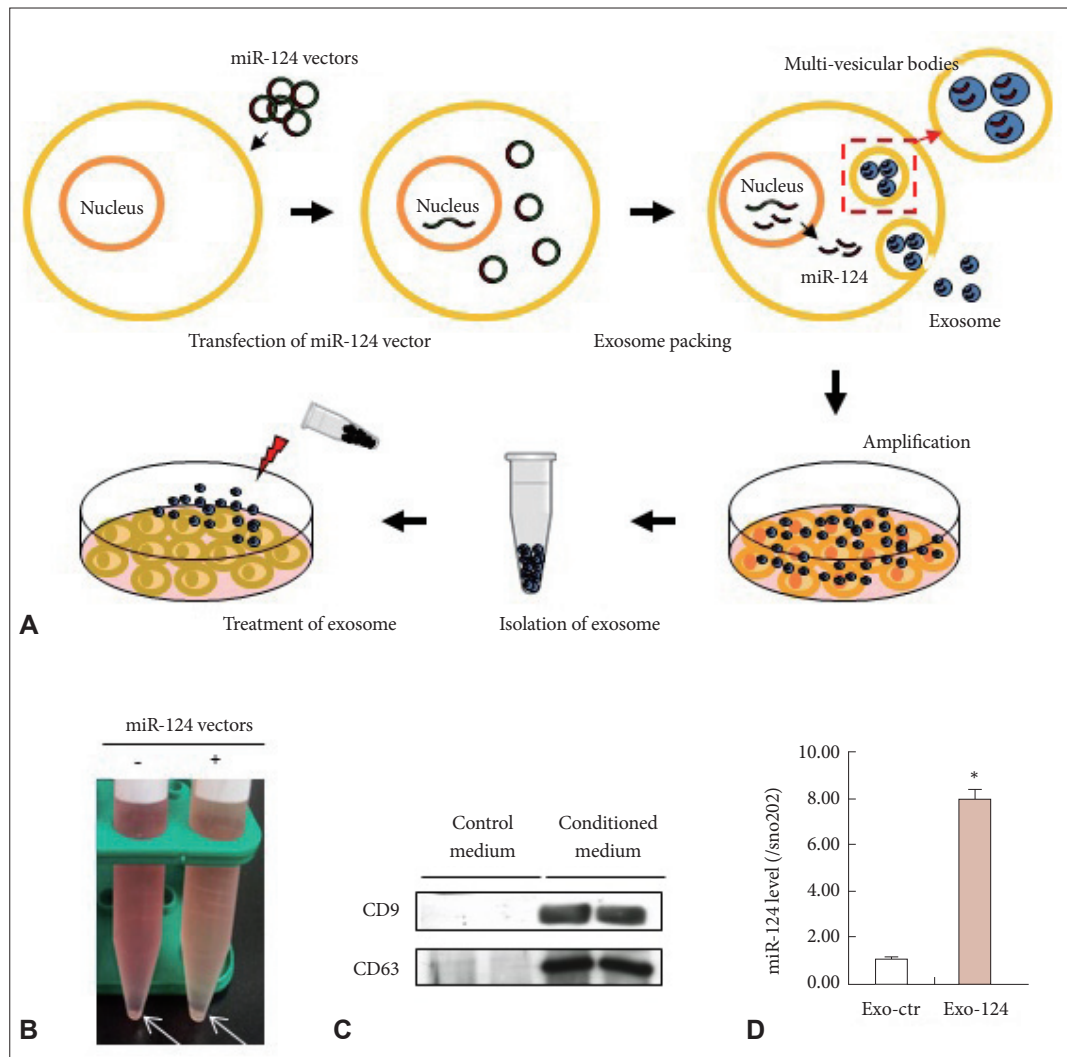


Figure 1. Generation of Exo-124. A: Diagram showing the procedure used to generate Exo-124. We repeatedly harvested Exo-124 from HEK 293 cells overexpressing miR-124. B: The harvested Exo-124 pellets. C: Expression of CD9 and CD63 in Exo-124. Exosomes harvested from the conditioned medium expressed CD9 and CD63, whereas exosomes from the normal control medium did not express these markers. D: Compared to the control exosomes (Exo-ctr), Exo-124 expressed a high level of miR-124, as measured by real-time PCR ($n = 3$ per group). * $p < 0.05$.

culture medium did not produce any CD9- and CD63-bearing materials. We thus confirmed the presence of exosomes.

We then measured the expression of miR-124 in the exosomes. Control exosomes (Exo-ctr) harvested from naïve HEK 293 cells that were not transfected with miR-124 expressed very low levels of miR-124. On the other hand, miR-124-enhanced exosomes (Exo-124) harvested from HEK 293 cells that were transfected with the miR-124 expression vector expressed much higher levels of miR-124 (Figure 1D). Thus, we moved forward to *in vitro* and *in vivo* applications of Exo-124.

To confirm intercellular transfer of the exosomes, we labeled the exosomes with PKH67 and added Exo-124 to the culture medium of HEK 293 cells for 24 h. After Exo-124 treatment, the PKH67 fluorescence of the recipient HEK 293 cells was confirmed by flow cytometry (Figure 2A, B, and C) and immunocytochemistry (Figure 2D). These results demonstrated that Exo-124 produced by our isolation and treatment protocol was efficiently taken up by recipient cells.

Because previous studies have demonstrated the efficacy of exosome-mediated delivery of miRNA,¹⁴ we proceeded directly to the therapeutic application

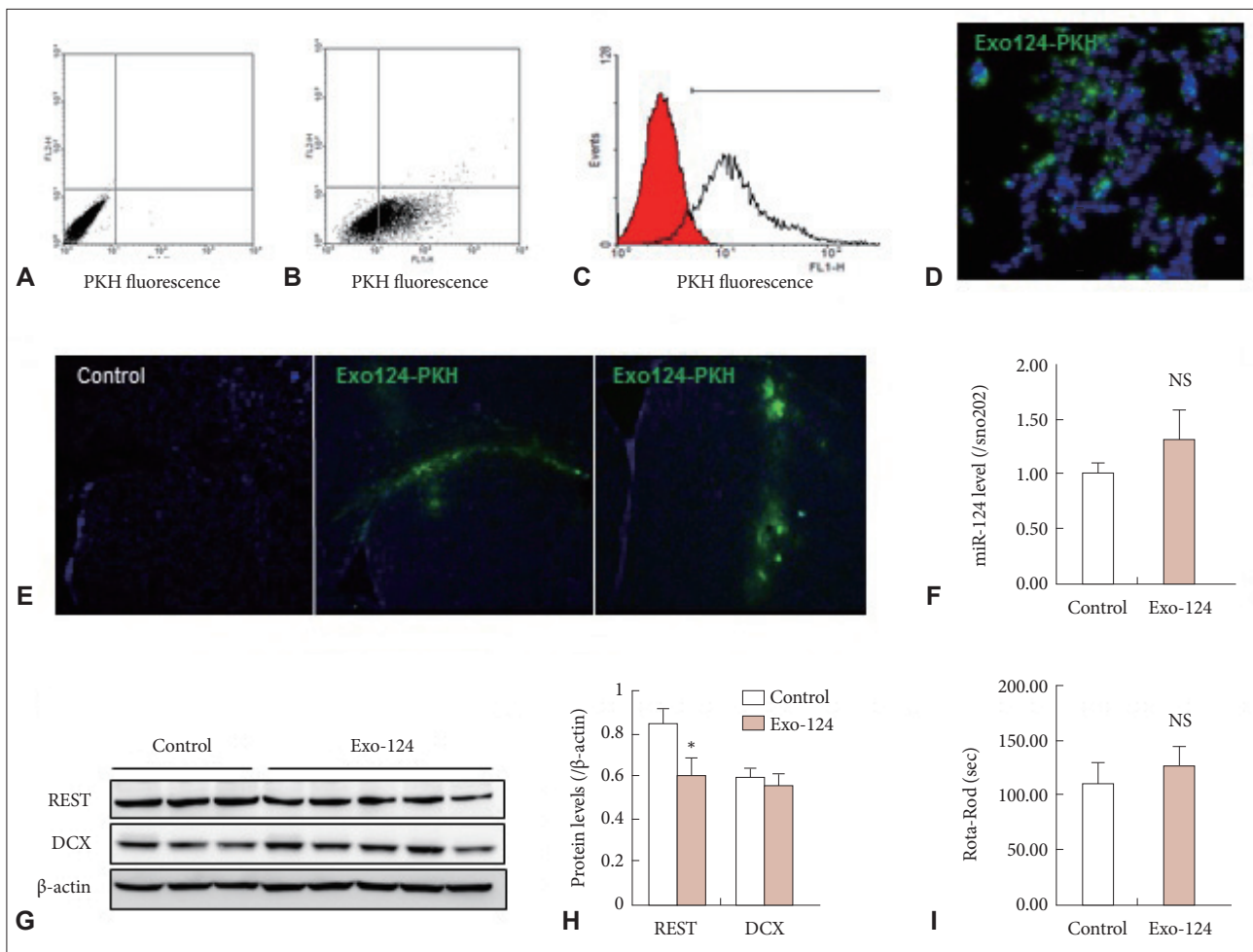


Figure 2. Delivery and therapeutic effects of Exo-124 in a HD model. A, B, and C: Exo-124 was labeled with PKH67. The labeled Exo-124 exhibited PKH67 fluorescence (B), whereas the unlabeled Exo-124 did not (A), as revealed by flow cytometry (C). D: When Exo-124 was added to the culture medium of HEK 293 cells, the cells exhibited PKH67 fluorescence. E: When Exo-124 was injected into the striatum of R6/2 HD mice, Exo-124 was taken up by the striatum and corpus callosum. F: One week after the injection of Exo-124, the level of miR-124 expression in the brains of the Exo-124-injected R6/2 mice was similar to that in the control mice, with a slight trend toward an incremental increase in miR-124 expression ($n = 3$ for the control and 5 for the Exo-124 group). G and H: The injection of Exo-124 significantly decreased REST expression in the R6/2 mice, as measured by western blotting (G) and densitometry (H) ($n = 3$ for the control and 5 for the Exo-124 group). However, the level of Dcx was not changed. I: The injection of Exo-124 also did not significantly affect Rota-Rod performance ($n = 5$ per group). * $p < 0.05$. HD: Huntington's disease, REST: RE1-Silencing Transcription Factor.

of Exo-124 in R6/2 HD transgenic mice. We injected Exo-124 into the striatum of 6-week-old R6/2 mice. Upon analysis 1 week later, PKH67-labeled Exo-124 had been taken up by the striatum (Figure 2E). We measured miR-124 expression in the mice and found that the Exo-124-treated R6/2 mice exhibited slightly higher levels of miR-124 expression compared to the control (non-treated) R6/2 mice, but this difference was not significant (Figure 2F). We hypothesized that the endogenous abundance of miR-124 in the brain masked the effect of delivery. Thus, we measured brain levels of REST protein, the key target protein of miR-124, and we found that REST expression was lower in the Exo-124-treated R6/2 mice than in the control R6/2 mice (Figure 2G and H). Next, because the miR-124-REST system regulates neurogenesis, we measured Dcx (doublecortin) expression in the brain and found that Exo-124 treatment had no effect on Dcx protein levels (Figure 2G and H). Moreover, at 7 weeks of age, no differences in Rota-Rod performance were observed between the Exo-124-treated R6/2 mice and the control R6/2 mice (Figure 2I).

DISCUSSION

In this study, we demonstrated the feasibility of exosome-based therapeutic delivery of miR-124 in a HD model. We could generate Exo-124 from a miR-124-overexpressing cell line, enabling stable and efficient generation of miRNA-bearing exosomes. In addition, when Exo-124 was delivered to the striatum, it reduced the expression of REST. However, Exo-124 had little effect on Rota-Rod performance.

The delivery method is one of the critical issues in the development of miR-based therapeutics for neurological diseases. When using antagomirs, the antisense inhibitor oligonucleotides of miRNAs, it is possible to use naked formulations and to deliver them via stereotaxic injections into the brain.²⁸ However, when miRNAs themselves are delivered, it is necessary to use additional methods to protect the miRNAs from degradation, such as lipid-based delivery systems, poly-(lactic-co-glycolic acid) particles, or viral vectors.²⁹ The exosome method is more physiological and less immunogenic, and if the exosomes are engineered to express specific surface ligands such as integrins, the exosome method can be used to selectively reach specific target cells.²³

Exosomes or extracellular vesicles can deliver not only miRNAs but also mRNAs, non-coding RNAs, cellular proteins, or even prions.³⁰ Under natural conditions, exosomes mediate cross-talk between neurons and other cells, such as microglia and immune cells.³¹ Exosomes regulate development and normal brain function and also contribute to synaptic plasticity and regeneration.^{23,32,33} In disease conditions, exosomes can be used to deliver RNAi, antigens for cancer immunotherapy, and therapeutic chemicals. In the present study, we showed that naïve miRNAs can be packed in exosomes and delivered as a therapeutic treatment for neurodegenerative diseases.

Although we showed that exosome-encapsulated miRNAs could be transferred to recipient cells *in vitro*, the current method must be further developed for *in vivo* applications. Because the therapeutic efficacy of Exo-124 was modest, it may be necessary to increase the dose of miRNAs packed in the exosomes. In addition, because the therapeutic effect of miR-124 might be limited, it is necessary to test whether the delivery of other miRNAs via the exosome method might have greater therapeutic effects. Candidate miRNAs might include miR-9, miR-22, miR-125b, miR-146a, miR-150, and miR-214.^{7,34-36} Moreover, differences in the tissue distribution and efficiency of target gene repression should be compared between systemic injections, such as intravenous or subcutaneous methods, and local injection for future *in vivo* applications. In this case, for targeted delivery to the brain, we can add integrin ligands or peptides that recognize transferrin receptors.^{37,38} Overall, the current study provides a proof of concept for exosome-based delivery of miRNAs to the brain, warranting further studies of this method.

Conflicts of Interest

The authors have no financial conflicts of interest.

Acknowledgments

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