miR-6780-5p-Enriched Exosomes Derived From Butylideneephthalide-Pre-Conditioned Human Olfactory Ensheathing Cells Via Autophagy Improve Motor Coordination and Balance in a SCA3/MJD Mouse Model

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Abstract

Background: The development of acellular products is a new trend for regeneration medicine. To provide an acellular product exhibiting characteristics of cells and usefulness as a therapeutic agent, exosomes were employed in the current studies. Method and Result: The therapeutic agent hsa-miRNA-6780-5p was enriched up to 98 folds in exosomes derived from butylidenephthalide (bdph)-pre-conditioned human olfactory ensheathing cells (hOECs) compared to naïve hOECs exosomes. The particle size of exosomes derived hOECs and exosomes derived hOECs pre-conditioned bdph were around 124.17 nm and 117.47 nm, respectively. The role of hsa-miRNA-6780-5p was first demonstrated in our studies using a liposome system, showing that it enhances autophagy and inhibits spinocerebellar ataxia type 3 (SCA3) disease proteins of polyglutamine (polyQ) tract expression. At the same time, the exosomes with enriched hsa-miRNA-6780-5p were further applied to HEK-293-84Q, thus resulting in decreased expressions of polyQ and increased autophagy in the cells. In contrast, the results were reversed when the autophagy inhibitor, 3MA, was added to the cells treated with hsa-miRNA-6780-5p enriched exosomes, indicating that the decreased polyQ expression was modulated via autophagy. The SCA3 mice showed improved motor coordination behavior when they received intracranially injected exosomes enriched with hsa-miRNA-6780-5p. The SCA3 mouse cerebellum tissue having received hsa-miRNA-6780-5p enriched exosomes also showed a decreased expression of polyQ and increased
expression of autophagy marker. **Conclusions:** Together, our findings provide an alternative therapeutic strategy for SCA3 disease treatment, using miRNA enriched exosomes derived from chemically pre-conditioned cells.

Keywords: miRNA, enriched exosomes, pre-conditioning, polyglutamine, spinocerebellar ataxia type 3, autophagy.

**Background**

Spinocerebellar ataxia type 3 (SCA3), also named Machado-Joseph diseases (MJD), is an autosomal dominant cerebellar ataxia. The disease is rare, with prevalence around 1–5/100,000 individuals. Mainland China has the highest prevalence of SCA3 with 62.6%, followed by Brazil with 59.6%, Japan with 43%, and Germany with 42% [1]. The highest prevalence of spinocerebellar ataxia (SCA) in Taiwan is type 3 with the level of 47.3% [2].

In SCA3, an expanded CAG repeat by more than 45 is found in the exon 10 of ATXN3, which is translated into a disease mutant ataxin-3 with expanded polyglutamine (polyQ) tract. A negative correlation between expanded polyQ and disease onset has been demonstrated [3]. Gait ataxia is the most common symptom of SCA3 and the average survival of individuals affected by SCA3 is 21.18 years after onset of symptoms [4], meaning the period they suffer from the disease. Until now, chemical drugs and stem cells are two types of active pharmaceutical ingredients (API) applied in the clinical trials of SCA3. Although many clinical trials have been performed for the treatment of SCA3, there is no U.S. Food and Drug Administration (FDA)-approved drug for the disease. Most therapeutic strategies for SCA3 patients are employed according to their symptom such as supportive treatment or physical therapy.
The main clinical symptom of SCA3 is volume decrease of the cerebellum, associated with a loss of Purkinje cells. Therefore, glial cells could be beneficial for the treatment of this neurodegenerative disease. The olfactory ensheathing cells (OEC), which are glia derived cells ensheathing the axons of olfactory receptor neurons, have shown promising results in this disease [5]. Human OEC can be obtained from surgical process of nasal polyps [6]. These cells have been applied to the treatment of spinal cord injury, with results showing increased glucose metabolic activity, SDF-1α and the enhancement of CXCR4 and PrPC in the treated versus untreated group [7]. Moreover, their ability to modulate neuroinflammation has also been demonstrated [8]. Prof. Chiou’s team has implanted OEC into the dorsal raphe nucleus of SCA3/MJD transgenic mice, showing an improvement in animal behavior and alleviated cerebellar inflammation [9]. Until now, cell transplantation therapies have been applied for the treatment of stroke and neurodegenerative diseases with promising results. Furthermore, a previous study revealed that the therapeutic component may come from the secreted factors instead of the cells themselves, providing a similar therapeutic efficacy [10].

Exosomes, which are secreted from cells, are around 100–200 nm in size. There are a lot of studies indicating that exosomes are an acellular product which may reduce tumorigenesis and display high potential for regeneration [11-13]. Various types of cell-derived exosomes have been used for the treatment of neurodegenerative diseases. For example, Schwann cell-derived exosome were found to regenerate peripheral neural system [14]; adipose-derived stem cell (ADSC)-derived exosome to repair damaged mitochondria in ALS disease [15]; or fibroblast, L-cell [16]-derived exosome to attract the autocrine Wnt10b from damaged neural cells to assist with axonal regeneration [17]. Until now, there have been at least 30 clinical trials using exosomes to treat disease such as chronic, pneumonia, neurodegenerative diseases [18, 19].
Another advantage of exosomes is their flexibility for therapeutic agent encapsulation, such as foreign drug encapsulation or using stimulation methods to amplify the therapeutic agent. To enhance yield of therapeutic agents in the exosomes secreted from cells, preconditioning chemical drugs have been utilized, similar to immune cell priming. Methods such as neutral and cationic-bare liposome addition, inhibition of glycolysis and oxidative phosphorylation, low level electrical stimulation, or adiponectin addition resulted in enhancement of exosome secretion from the parental cells. Enhancement of autophagy may improve the disease progress. Beclin-1, an initiator of autophagy involved in an mTOR-dependent pathway, present lower expression in the patient’s fibroblast. In addition, the ratio of LC3II/ LC3I is also lower in patients. The chemical agent Butyridenecephthalide (Bdph) has been demonstrated to induce autophagy in cells. Therefore, a new strategy using Bdph to trigger glial cell production of exosomes stimulating autophagy was investigated in the current study. And moreover, Bdph-magnified therapeutic hsa-miRNA in exosomes were as a delivering therapeutic agent for SCA3 treatment.

Results

Exosome characterization

To confirm exosome production quality, the total protein and particle size were analyzed for each batch. We performed three times the same procedure for exosome production in the hOECs, with or without Bdph treatment. The average concentration of exosomes derived from hOECs without Bdph (OECexo) or with Bdph (BPPexo) treatment were 9.03 and 8.78 µg/ml using the BCA kit analysis. The average size of exosome from the hOECs without Bdph and with Bdph treatment performed were 124.17 ± 11.91 nm and 117.47 ± 10.09 nm.
by NTA measurement, respectively. (Figure 1a). The two exosome populations were well-
distributed as shown in Figure 1b and c.

Bdph increases the autophagy element production in exosomes and their treatment in
HEK-293-84 Q cells

To confirm whether autophagy markers can be secreted from hOECs cells pre-conditioned
with Bdph, the expression of two markers, beclin1 and LC3I/LC3II, was analyzed. The total
proteins served as an internal control for each group of exosomes. The exosomes derived
from the glial cells, hOEC, treated with Bdph (BPPexo) had increased expression of the
autophagy markers, Beclin1 and LC3I/LC3II, compared with exosomes derived from naïve
hOECs without Bdph (OECexo) (Figure 2a).

The two exosomes, BPPexo and OECexo, were further treated in the HEK-293-84Q cells to
confirm their function in regulating expression of polyQ protein. The results showed that the
disease protein polyQ was decreased when HEK-293-84Q cells were treated with BPPexo
(Figure 2b). However, the expression of polyQ in HEK-293-84Q cells did not show any
difference whether they received OECexo or PBS (untreated) (Figure 2b). We speculate that
this outcome may result from an increase of autophagy in the cells. The results showed that
the expression of Beclin1 was increased in the cells upon BPPexo treatment, and not in the
group of OECexo or untreated (Figure 2b). To confirm the effect of exosomes on autophagy,
the autophagy inhibitor drug 3MA was added to HEK-293-84Q cells to block the pathway of
autophagy. The expression of Beclin-1 was inhibited when the cells received the 3MA drug
compared to cells without 3-MA treatment (Figure 2b). We found that the expression of
polyQ was increased when HEK-293-84Q cells received both 3MA and BPPexo (Figure 2b).
These results indicate that the exosome of BPPexo play the role in autophagy pathway and
thus BPPexo did not have any effect on HEK-293-84Q cells when the autophagy pathway was inhibited. These findings imply that the exosomes derived from Bdph treated hOECs were able to encapsulate a therapeutic agent related to autophagy.

The miRNA microarray of hOEC cells treated with Bdph or without Bdph was analyzed to screen for candidate miRNA. The changes above three-fold intensity were included in the panel (Figure 2c). In addition, the panel of miRNA in the Figure 2c was further narrowed to key candidate miRNAs related to the mTOR pathway. According to the online tool of microT-CDS and TargetScan Release 7.2, miRSystem, and miRDB, four miRNAs were selected. The expression of four miRNAs, Has-miRNA-6780-5p, Has-miRNA-1275, Has-miRNA-6126, and Has-miRNA-29b-3p, related to the mTOR pathway, were further confirmed in the exosomes.

The expression of the four candidate miRNAs was further analyzed from the exosomes of BPPexo and OECexo. The results showed that the expression of Has-miRNA-6780-5p and Has-miRNA-6126 have 98.62 and 86.97 fold increases in the BPPexo compared to the OECexo, respectively (Figure 2d), whereas the Has-miRNA-1275 and Has-miRNA-29b-3p only had 5.05- and 2.57-fold increases compare to exosome without Bdph. The Has-miRNA-6780-5p and Has-miRNA-6126 were two miRNA candidates to use for examining outcomes on autophagy and polyQ expression.

Introducing an autophagy enhancer, hsa-miRNA 6780-5P, via a liposome system into HEK-293-84Q cells

Before screening the potential miRNA candidates for polyQ decrease and autophagy, we used a homemade liposome system to confirm miRNA delivery efficiency. The miRNA-Fan acted as model miRNA based on its fluorescence, with excitation at 493 nm and emission at
517 nm, reporting a successful miRNA delivery with fluorescence in the cells. The liposomal system has DiL dye fluorescence with red color upon successful delivery in the cells. The results showed that the cells presented with red color fluorescence after treatment for one hour, meaning that the liposomes were uptake by the cells (Figure 3a). At the same time, the cells also showed green color fluorescence, meaning that the miRNA-Fan were in the cells (Figure 3b). The two fluorescence images were merged to confirm colocalization of liposome and miRNA. The colocalization of liposome and miRNA is presented in the merge image (Figure 3c), confirming that the miRNA has been delivery by liposomal system into the cells.

To confirm whether the hsa-miRNA 6126 and hsa-miRNA 6780-5p play the role in autophagy, the online tools microT-CDS and TargetScan Release 7.2, miRSystem, and miRDB were employed. The hsa-miRNA 6126 and hsa-miRNA 6780-5p were shown affinity in binding with mRNA of PI3KR5 or MLST, or CAP1, which related to the mTor pathway. To confirm the inhibition efficacy on the mRNA of PI3KR5, MLST, or CAP1 through the hsa-miRNA 6126 and hsa-miRNA 6780-5p, the miRNA was delivered into HEK-293-84Q cells via homemade liposome. The results showed that only PI3KR5 was significantly inhibited by the hsa-miRNA 6126 or hsa-miRNA 6780-5p compared to CAP1 and MLST8 (Figure 4a). Moreover, to confirm the hsa-miRNA 6126 or hsa-miRNA 6780-5p in the inhibition in the mRNA of ATXN3, two miRNAs were examined in the HEK-293-84Q cells, respectively. The results showed that no significant inhibition of ATXN3 mRNA is observed in HEK-293-84Q cells from hsa-miRNA-6780-5P, whereas a slight decrease of ATXN3 results from hsa-miRNA-6780-5P (Figure 4a). Two candidate miRNAs were further used to confirm inhibition of polyQ expression in HEK-293-84Q cells. The expression of polyQ decreased significantly when HEK-293-84Q cells were treated with hsa-miRNA 6780-5p (Figure 4b). In contrast, the expression of polyQ did not decrease after HEK-293-84Q was added to hsa-miRNA-6126 (Figure 4b) even though hsa-miRNA-6126 resulted in a slightly
decrease of ATXN3. Expression of the autophagy marker Beclin1 was confirmed in HEK-293-84Q cells upon hsa-miRNA 6780-5p treatment. The results show that the expression of Beclin 1 was increased dose-dependently when cells were treated with hsa-miRNA 6780-5p (Figure 4c). We further confirmed that the expression of another autophagy marker, LC3I/LC3II, was increased when HEK-293-84Q cells were treated with hsa-miRNA 6780-5p/liposomes, whereas the liposome alone or hsa-miRNA 6780-5p without liposome carried did not increase the expression of LC3I/LC3II (Figure 4d). To prevent a potential effect of the liposome alone or hsa-miRNA 6780-5p alone on the expression of polyQ, they were also added to the HEK-293-84Q individually. The inhibition of polyQ was observed in cells only with hsa-miRNA 6780-5p/liposome group, contrary to the groups of hsa-miRNA 6780-5p alone, liposome alone, or PBS (Figure 4d). These finding indicate that hsa-miRNA 6780-5p play a role in enhancing autophagy and decreasing the expression of polyQ.

Exosome treatment in the SCA3 mouse model

Based on the above results, the exosomes derived from naïve hOEC or pre-conditioned with Bdph were applied into SCA3 mice to confirm the efficacy of exosome treatment. The exosomes from hOECs with (BPPexo) or without Bdph (OEC exo) priming were injected into the SCA3 animal. The low and high dose of exosomes derived from hOECs with Bdph priming were also examined in the study. The behavior of the animals on the rotarod was assessed to evaluate the balance and coordination after treatment. To determine the treatment efficacy, untreated SCA3 and wild-type animals were compared in the study. We found that the latency to fall in the wild-type mice was around 150 seconds in the rotarod test, whereas it was below 100 seconds in untreated SCA3 mice. The results further showed that SCA3 animals receiving a high dose of BPPexo stayed longer on the accelerating rotating rod for an average above 120 seconds compared to below 100 seconds for the untreated group.
(Figure 5a). The group receiving a low dose of BPPexo showed they can stay above 120
seconds on the accelerating rotating rod in the first three weeks, which was then decreased
after three weeks of injection (Figure 5a). The SCA3 animals received OEC exosomes (OEC
exo) displayed an increased time on the accelerating rotating rod in the first two weeks of
treatment, with 122 seconds on the rod, but their latency to fall was decreased like the
untreated group (Figure 5a). The results indicate that both exosomes were effective, with the
higher dose of BPPexo resulting in a longer period of therapy. The same dose of two
exosomes resulted in different efficacy, suggesting different therapeutic mechanisms for the
two exosomes. Lastly, we further confirmed the expression of polyQ and autophagy using
brain tissue. Brain tissue was collected from the animals after 6 weeks of treatment, at the
age of 20 weeks, to confirm the expression of polyQ and autophagy. The wild-type animals
showed less extended polyQ expression at 63 kDa, whereas the untreated SCA3 mice showed
overexpression of polyQ (Figure 5b). The expression of polyQ at 63 kDa was decreased when
the SCA3 mice received a high dose of BPPexo compared with to low dose of BPPexo or
high dose of OEC (Figure 5b). To confirm whether the treatment efficacy resulted from the
autophagy in mice, the autophagy marker, LC3B, was analyzed from the brain tissue. The
results showed that wild-type animals had more LC3B expression compared to the untreated
SCA3 group (Figure 5c). The expression of LC3B following the high dose of BPPexo showed
higher expression in the brain than those of untreated group and slightly higher that of
OECexo (Figure 5c). Therefore, these results reveal that BPPexo has a potential in treating
SCA3 disease via a therapeutic mechanism involving autophagy.

Discussion

The strategy of using miRNAs, such as miR-25 [28], miR-9, miR-181a, and miR-494 mimics
[29] for SCA3 therapy has been widely studied. These miRNAs were shown to directly target
the mRNA of ATXN3. However, these strategies also result in inhibition of normal ATXN3, indicating that some patient with heterozygous ATXN3-SCA3 may be not suitable to receive this treatment strategy. The downregulated autophagy has been measured in SCA3 patients [25, 26, 30]. According to previous studies on current therapeutic targets for SCA3 therapy, the destination of those strategies and related mechanisms for SCA3 treatment would go to autophagy enhancement [31].

The miR-6780-5p plays a role in promoting ovarian cancer metastasis in the previously studies [32]. The role of miR-6780-5p in autophagy has not been discussed yet. We found that miR-6780-5p inhibits the expression of PIK3R5 and increases the expression of LC3I, LC3II, and Beclin1 (Figure 4). The PI3KR5 inhibitor has been used in previous studies as an enhancer of autophagy. For example, the miR-210-5p, PI3KR5 inhibitor has been demonstrated to activate oncogenic autophagy in osteosarcoma cells [33]. Therefore, using miRNA related to AKT/mTOR signaling pathway provides an alternative strategy for autophagy enhancement.

We found that exosomes carry autophagy induction elements, in which the exosomes were derived from hOECs pretreated with Bdph (Figure 2). The hsa-miR-6780-5p, a PIK3R5 inhibitor, was overexpressed for 98-folds in the exosomes derived from hOEC cells after Bdph treatment. Moreover, hsa-miR-6780-5p also acts as an autophagy enhancer in our studies. Lee and colleagues have shown that cells receiving Bdph have increased autophagy [27]. Combination of the previously results and our studies, the exosomes would carry the elements from the parental cells which means the exosomes could as carrier for therapeutic agent.

Exosomes exhibiting the derivative of cells and cell-free nanoscale particles have shown their potential in disease treatment compared to cell alone treatment strategies [34]. Therefore, the exosomes encapsulated with overexpression of miR-6780-5p were injected into SCA3 mice
for an evaluation of treatment efficacy in the current study. The results showed an improved motor balance compared to mice receiving naïve exosomes or no treatment. Hsieh and colleagues have implanted intracranial with hOECs the same SCA3 mouse model at 13 weeks of age. The average latency to fall was 130–150 seconds in the treated group compared to 95–125 seconds in the untreated group between 15 and 24 weeks of age [9]. In our studies, the average latency fall was of 130–146 seconds in the exosomes derived from Bdph primed hOECs treated group compared to 97.5–115 seconds in the untreated group from 15 to 20 weeks of age (Figure 5). Although efficacy was similar for the two treatments, the underlying therapeutic mechanism is different. Hsieh and colleagues found increased levels of tryptophan hydroxylase 2 and ryanodine receptor (RYR) in the brain after hOECs treatment [9]. In our studies, we found elevated levels of autophagy markers in the SCA3 mice after receiving exosomes derived from Bdph primed hOECs. Therefore, exosomes are considered as potential drug carriers, hence providing another strategy using drugs to enhance therapeutic agent expression and act as a biomarker to evaluate the disease treatment efficacy.

Conclusion

In conclusion, the proposed therapeutic mechanism for the treatment of SCA3 via exosomes derived pre-condition medium of hOECs is summarized in Figure 6. The has-miR-6780-5p was firstly demonstrated when evaluating autophagy in the disease cell model HEK-293-84Q and showing efficacy in decreasing polyQ expression in our study. We also found that Bdph, an autophagy enhancement drug, elevates the production of has-miR-6780-5p in exosomes. Most importantly, the elevated has-miR-6780 in the exosomes was further employed to treat SCA3 mice. The results showed an improved motor coordination in the SCA3 mice received exosomes with elevated has-miR-6780 compared to naïve exosomes.
Materials and Method

Cell and exosome production

Human olfactory ensheathing cells (hOECs) obtained from patients (IRB NO. IRB105-144-A) were provided by the Lab of Prof. Shinn-Zong Lin. The hOECs were maintained in medium of DMEM/f12 (Hycolne) provided 10 % defined fetal bovine serum (FBS; Hyclone) and 1% penicillin/streptomycin (100 units/L Penicillin G and 100 μg/L streptomycin sulfate, GIBCO). The exosome production started with the cell seeding density reached 1.2 × 10^4/cm^2 followed by replacement of the Hycolne FBS with a commercial exosome depletion FBS medium (GIBCO). For priming studies, 10 and 50 μg/ml Bdp were mixed with medium after cell seeded for 24 hours. The conditioned medium was harvested for immediate EV purification after another 48 hours in culture. The harvested condition medium was filtered with 0.2 μm filter (regenerated cellulose) to remove cell debris followed by a 10 kDa ultrafiltration membrane to concentrate the medium. The exosome purification kit—ExoQuick™ was used by adding 1/5 volume of concentrated conditioned medium. Finally, the exosomes were resuspended in PBS and stored at −80 °C for further analysis.

Liposome preparation

The cholesterol and lipid of DPPC, TAP18, DSPCPEG2000, or dye of DiL were dissolved in chloroform (10 mg/ml) in a round bottom flask. The flask was then connected to a rotary evaporator (N-1300, EYELA) and maintained with temperature at 25 °C using a water bath. When the chloroform was removed, a homogeneous lipid film was formed on the walls of the flask. The residual chloroform was then to evaporate using 5 × 10^−4 Ttorr vacuum system.
for at least one hour. The lipid film was then hydrated with PBS by sonication in a water bath at 24 °C. The liposome dispersion was then extruded by a Mini-Extruder (Whatman, Inc., Clifton, NJ, USA) using 0.2 µm polycarbonate filters at room temperature. The extruded liposomes were kept at 4 °C for at least one hour and then stored at −20 °C for further experiments.

Microarray miRNA gene profile from hOEC cells
To obtain miRNA of cells with or without Bdph treatment, the total Exosome RNA & Protein Isolation Kit (Thermo Fisher Scientific Inc.) was used. Briefly, the cell lysate was added one volume of 2X denaturing solution and mixed thoroughly. One volume of acid-phenol: chloroform was added to each sample followed by a glass-fiber filter to obtain the total miRNA. The analysis of miRNA gene profile was contracted to GeneDireX Inc.

Quantification of miRNA in the exosomes via real-time PCR
The sequences of primers for has-mir-6126, has-mir-1275, has-mir-29b-3p and has-mir-6780 were respectively: gtgaaggccgcgggaga, gtgggggagaggctgtc, tagcaccatttgaaatcagtgtt and tggggaaggcttggcagggaaga (Genomics).
For the isolation of microRNAs, the Total Exosome RNA & Protein Isolation Kit (Thermo Fisher Scientific Inc.) was used to isolate miRNAs. Briefly, 200 µl of dissolved exosomes were supplemented with 200 µl Denaturation Buffer and then purified by phenol: chloroform and a filter cartridge. The extracted miRNAs were eluted in 100 µl Elution Buffer. Reverse transcription and qPCR of microRNAs was conducted using the Applied Biosystems kit. Mir-X™ miRNA First-Strand Synthesis and TB Green qRT-PCR kit (TAKARA BIO USA, Inc.) were used. The 100-µl reaction solution contained 5 µl mRQ Buffer (2×), 3.75 µl RNA Sample (0.25–8 µg), 1.25 µl 10×RT mRQ Enzyme, 90 µl ddH₂O. The reactions were
carried out at 37°C for 60 min and at 85°C for 5 min to inactivate the enzyme. In a 25-μl reaction, 2 μl cDNA was mixed with 9 μl ddH₂O, 12.5 μl TB Green Advantage Premix (2×), 0.5 μl ROX Dye (50×) and 0.5 μl miRNA primer (has-miR-6126, has-miR-6780-5p, has-miR-1275, and has-miR-29b-3p). The human u6 specific primer act as internal control and the prepared procedures were the same as above except that 0.5 μl of u6 forward primer (5′-ggaacgatacagagaagattagc-3′) and 0.5 μl of u6 reverse primer (5′-tggaacgcttcaggaatttg-3′) were used. To prevent false-positive results, a negative control containing nuclease-free water instead of the template miRNA was also used. QPCR reaction was performed at 95°C for 10 s with one cycle, and then at 95°C for 5 s and 60°C for 20 s for 40 cycles, followed by a final cycle at 95°C for 60 s, 55°C for 30 s, and 95°C for 30 s. Data were analyzed by the QuantStudio™ Design & Analysis Software.

Characterization of exosome

The purified exosomes were precipitated by ExoQuick™ to obtain the exosome pellet and then resuspended with RIPA lysis buffer. The concentration of protein was assessed using the bicinchoninic acid (BCA) Protein Assay Kit (Merck-Millipore). The 10 μg of protein was loaded into 12% TGX FastCast Acrylamide Solutions (Bio-Rad Laboratories, Inc.). The TGX FastCast gels were imaged with Fusion Solo S (Vilber) and then transferred to a PVDF membrane. The membranes were blocked with 5% low-fat milk powder suspended in 0.05% tween 20 in PBS for one hour at room temperature. Membranes were then probed for exosome proteins with LC3B and BECN1. These primary antibodies were subsequently probed with appropriate secondary antibodies conjugated to horseradish peroxidase. Enhanced chemiluminescent (ECL) HRP substrate was added and chemiluminescence was detected using the Fusion Solo S image analyzer (Vilber).
Nanoparticle tracking analysis (NTA)

Nanoparticle tracking analysis was used to determine the size and particle concentration of exosomes or liposomes using a Nanosight LM10 (Malvern Instruments; Malvern, United Kingdom). The exosomes or liposomes were diluted to 1–2 μg/mL or 10–20 ng/mL in PBS, and 3 videos of 30 s were acquired for each triplicate.

Encapsulation of miRNA with liposomes

Three synthetic double strand miRNA mimics were used. The sequence of has-miRNA-6126 (Biotools Co., Ltd., Taipei, Taiwan) is forward: 5′-gugaaggccgggaga-3′, reverse: 5′-uccgccccuucacuu-3′, has-miRNA-6780-5p (BIOTOOLS Co., Ltd., Taipei, Taiwan) is forward: 5′-uggggaaggcuuggcagggaaga-3′, reverse: 5′-uucccugccaagccuuccccauu-3′, and acrofan duplex (GeneDireX, Inc.) is forward: 5′-uucuccgaacgugucacgutt-3′, reverse: 5′-acgugacaguuccaggaatt-3′. The synthetic double strand miRNA mimics were mixed with appoint concentration of miRNA into the liposome solution (25 liposomal particles/cell). The liposome solutions were heated with a thermoheater at 42˚C for 10 minutes. The miRNA solutions were added to the heated liposome solutions immediately and then mixed with an ultrasonic bath. The well mixed miRNA/liposome solutions were kept on ice for 5 minutes before further studies.

Disease model cell

The HEK 293-84Q-GFP artificial disease model was a gift from the lab of Prof. Tzyy-Wen Chiou. The original cell lines of HEK 293T were acquired from Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). The plasmid was purchased from Addgene Co. (Cambridge, MA, USA) followed by lentivirus transfection kit to transfec the plasmid into the cells. The cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) with 10%
FBS (GIBCO) and 1% penicillin/streptomycin (Hyclone, Logan, Utah, USA) in an incubator supplying 5% CO\textsubscript{2} at 37 °C.

**Liposome/exosome treatment in the HEK 293-84Q and NIH-3T3 models**

The HEK 293-84Q-GFP cells were seeded to 1 × 10\textsuperscript{6}/well into 6 well cm dish for overnight cultivation. The exosome (30 μg) or liposome/miRNA (above described method) was added to the cells within the medium. The exosome or liposome treated cells were analyzed via microscopy, Western blot or RT-PCR.

**Evaluation of HEK 293-84Q via Western blot**

The harvested cells were treated with RIPA buffer supplied with protease inhibitors for cell lysis. The lysed cells were further analyzed by Western blot. Then, the protein samples were centrifuged and the supernatant was stored at –80 °C. The denatured proteins were separated using 12% SDS-PAGE, and the separated proteins transferred to PVDF membranes (Merck-Millipore). The membranes were incubated with specific antibodies against polyQ (clone,1C2), Beclin1 (abnova, PAB12474), LC3B (abcam, ab51520), and GAPDH (Millipore, ABS16). The immunoreactive bands were detected using horseradish peroxidase-conjugated secondary antibodies generated in mouse (Millipore, AP124P) or rabbit (Millipore, AP132P) and ECL reagents (Merck-Millipore). The immunoreactive bands were analyzed using a luminescent image analyzer (FUSION Solo S, Vilber)

**Q-PCR for examination of mRNA expression**

The primer pair of CAP1 is: forward 5′-ggtggagtcaacctgtg-3′, reverse 5′-atccgcatctgaggctca-3′; the primer pair of ATXN3: forward primer 5′-
tggagtcctctccacagag- 3’, reverse primer 5’-ttcaggtgattggtccacagca- 3’; the primer pair of PI3KR5: forward 5’-gtgttgtgtgttcctgtgc- 3’, reverse 5’-ccatgaccttctgtcagcag- 3’; the primer pair of MLST8: forward 5’-cagtgcttggcttggcagtcagtcagtcag- 3’, reverse 5’-ttgttgacgccgtcgtagctga-3’; the primer pair of internal control (β-actin): forward with 5’-catgtacgtgtgccttgctatccaggc-3’ reverse with 5’-ctccttaatgtcacgcacgat-3’. All the primer pairs were purchased from Genomics.

To analyze RNA expression, total RNA was isolated from cells by using AllPure Total RNA Isolation Kit (AllBio Science Inc.), following the manufacturer’s protocol. The extracted mRNAs were eluted in 15 µl nuclease-free water. cDNA was prepared by using a 2-step method. First, an RNA mix was prepared with 1.5 µg of total RNA, and 1 µl of Anchored Oligo (dT) primer, mixed to a final volume of 9 µl with nuclease-free water and incubated at 70 °C for 2 min. Next, a second master mix of 10 µl of 2xES Reaction Mix, and 1 µl AllScript RT/RI Enzyme Mix was added to the RNA mix. The 20-µl reaction was then performed at 37 °C for 60 min and then at 85 °C for 10 min.

The 10-µl reaction Real-time quantitative RT-PCR was next prepared by using 5 µl of ORA™ SEE qPCR Green ROX L Mix, 0.5 µl of 500 nM target primer (CAP1, ATXN3 PIK3R5 MLST8), 3.5 µl of nuclease-free water, and 1 µl of cDNA. The following cycling conditions were used: 1 cycle of 2 min at 50 °C, 1 cycle of 2 min at 95 °C, 40 cycles of 5 s at 95 °C and 20 s at 60 °C, 1 cycle of 90 s at 95 °C, 1 cycle of 30 s at 55 °C, and 1 cycle of 30 s at 95 °C.

qRT-PCR results were analyzed by using QuantStudio™ Design & Analysis Software as the mean of normalized expression.

Animals

The female SCA3/MJD transgenic mice on a C57BL/6 genetic background, were purchased from the Jackson Laboratory. The mice were bred in NARLabs (Taipei, Taiwan) and
transported to the animal facilities of the National Dong Hwa University (Hualien, Taiwan) at 6–7 weeks of age. In the project, female wild-type C57BL/6 mice were purchased from Biolasco (Taiwan). All the animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC No. 106010) of National Dong Hwa University, and were conducted in accordance with the animal experiment guidelines of National Dong Hwa University.

Animal behavior

According to previous studies from the Lab of Prof. Tzyy-Wen Chiou [35], the rotarod test was performed four times prior to the treatment (intracranial route), and the recorded measurements were used as baseline values. After the treatment, the rotarod test was performed once a week for another 7 weeks (until the mice were 20 weeks of age). A rotarod apparatus (IITC, BioLASCO, Taiwan) were used to evaluate the motor coordination of the mice. The evaluation condition was performed under continuous acceleration at 4–40 rpm for 5 min, with at least six trials for each animal. The latency to fall time of each animal from the rod was also recorded for further analysis.

Exosome delivery to SCA3 by intracranial route

To evaluate the therapeutic efficacy of the exosomes derived from hOEC, with or without Bdph priming for SCA3, an intracranial delivery was performed. At 13 weeks of age, SCA3 mice were randomly divided into treated group and the untreated group. The exosome treatment in SCA3 was administrated with naïve hOEC exosome 178 μg (based on protein weight) as vehicle control and Bdph priming hOEC exosome by dosage of 89 and 178 μg. Wild-type C57BL/6 mice served as health controls. The mice were anesthetized with isoflurane (dose with scale at 1–2.5) and placed in a stereotaxic apparatus. The cranium was
drilled to generate 0.8 mm diameter burr holes at 0 mm lateral and 3.5 mm caudal to the Bregma. The exosome was injected using a 26s/2”/3 Hamilton syringe (Hamilton, Reno, NV, USA) and the syringe was inserted with depth of 2–3 mm through the dura into the meninges over the superior colliculus. The exosomes suspended in 10 μL of PBS were slowly injected with a rate of 1μL/min.

Evaluation of brain tissue samples

All the mice were sacrificed at 20 weeks of age. Cerebellar tissues were incubated on ice in RIPA buffer supplemented with protease inhibitors (cOmplete Protease Inhibitor Cocktail Tablets, Roche). Then, the protein samples were centrifuged and the supernatant was stored at −80 °C. The denatured proteins were separated using 12% SDS-PAGE, and the separated proteins transferred to PVDF membranes (Merck-Millipore). The membranes were incubated with specific antibodies against PolyQ (clone,1C2), Beclin1 (abnova, PAB12474), LC3B (abcam, ab51520), and GAPDH (Millipore, ABS16). The immunoreactive bands were detected using horseradish peroxidase-conjugated secondary antibodies generated in mouse (Millipore, AP124P) or rabbit (Millipore, AP132P) and ECL reagents (Merck-Millipore). The immunoreactive bands were analyzed using a luminescent image analyzer (FUSION Solo S, Vilber).

Statistical analysis

The data were analyzed using two tailed student's t-test. A statistically significant difference was reported when the p value was below 0.05. Data are presented as mean ± standard deviation (SD).
Figure legends

(a) Table

| Item    | Bdph (µg/ml) | Exosome Conc. (µg/ml)<sup>a</sup><sup>b</sup> | Size (nm) (NTA) |
|---------|--------------|---------------------------------------------|-----------------|
| OECexo  | 0            | 9.03±1.53                                   | 113.0±3.5 nm    |
| BPPexo  | 50           | 8.78±1.24                                   | 128.9±0.7 nm    |

<sup>a</sup>: Volume of harvested condition medium; <sup>b</sup>: Analyze by Bio-Rad protein assay

Figure 1. Exosome characterization. (a) Quantification of exosomes by BCA kit for protein concentration, and by NTA for size measurement. Particle distributions of (b) exosomes derived from hOECs and (c) exosomes derived from hOECs pre-conditioned with Bdph through NTA analysis.
Figure 2. Analysis of exosomes and their derived hOECs parental cells. (a) Expression of Beclin1 (BECN1) and LC3I/II in the exosomes derived from the hOECs treated with 0, 10, or 50 μg/ml Bdph. (b) Beclin1 (BECN1) and polyQ expressions in the HEK-293-84Q treated with hOECexo or BPPexo in the 3MA (autophagy inhibitor) or without 3MA conditions. BPPexo mean exosomes derived from hOEC cells primed with 50 μg/ml Bdph, OECexo mean exosomes derived from naïve hOEC cells. (c) Microarray analysis measuring the intensity of miRNA increase in the hOEC cells after 50 μg/ml Bdph treatment, in which the intensity was normalized to the hOEC without Bdph treatment. (d) Fold change of the miRNA expressions in the exosomes derived from hOECs treated with 50 μg/ml Bdph, normalized to the exosomes derived from naïve hOECs. The four candidate miRNAs were
selected based on their association with mTOR signal (DIANA TOOLS).

Figure 3. Homemade liposomal system for miRNA delivery. The homemade liposome presenting: (a) red fluorescence delivering model miRNA of miRNA-Fan, or (b) green fluorescence into the NIH-3T3 cells. (c) The merged image shows the colocalized fluorescence of liposome and miRNA.
Figure 4. Candidate miRNA screening. (a) The knock-down mRNA expressions of CAP1, ATXN3, PI3KR5, and MLST8 by has-miRNA 6126 delivered via homemade liposome (abbreviation as miRNA 6126), or has-miRNA 6780-5P delivered via homemade liposome (abbreviation as miRNA 6780), or homemade liposome alone (vehicle) in the HEK-293-84Q cells for 24 hours. *: p < 0.05, **: p < 0.01. (b) The expressions of polyQ in the HEK-293-84Q cells when the cells were treated with has-miRNA 6126 or has-miRNA 6780-5p delivered by homemade liposome. (c) The expressions of Beclin1 (BECN1) in the HEK-293-84Q cells when the cells were treated with various concentration of has-miRNA 6780-5p delivered by homemade liposome. (d) The expressions of polyQ and LC3I/LC3II in the HEK-293-84Q cells when the cells treated with has-miRNA 6780-5p delivered by homemade liposome.
Figure 5. Animal behavior study and brain tissue analysis. (a) Rotarod analysis in wild-type mice and SCA3 mice with or without exosome treatment. BPPexo means exosome derived from hOEC cells primed with 50 µg/ml Bdph. OECexo means exosome derived from naïve hOEC cells. **: p < 0.01 in comparison with untreated group. The expression of (b) poly Q and (c) autophagy marker, LC3B, in brain tissue from wild-type mice and SCA3 mice with or without exosomes treatment. BPPexo indicates exosomes derived from hOEC cells primed
with 50 µg/ml Bdph, OECexo exosome derived from naïve hOEC cells, and H and L the dose of exosomes with 178 µg and 89 µg.

Figure 6. Illustration of the mechanism underlying treatment with enhanced autophagy elements of miRAN 6780-5P derived from hOECs in SCA3 mice. The miRNA 6780-5P are proposed to be enriched in exosomes through Bdph stimulation of hOEC cells.

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Authors’ contributions

Yu-Shuan Chen, Horng-Jyh Harn, and Tzyy-Wen Chiou designed experiments, Yu-Shuan Chen, Zhen-Xiang Hong, Yi-Chen Huang, Yi-Tung Lin, Hui-Xuan Zheng, Pei-Yu Chen, and Hsueh-Hui Yang performed experiments. Yu-Shuan Chen, Horng-Jyh Harn, and Tzyy-Wen Chiou contributed to data compilation and paper preparation. Horng-Jyh Harn, Peir-Rong Chen, Hsieh-Chih Tsai, Shinn-Zong Lin, Tsung-Jung Ho, and Tzyy-Wen Chiou provided critical feedback on the study and contributed to the preparation of the paper. All authors listed reviewed the paper and provided feedback. All authors read and approved the final manuscript.

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DISCLOSURE

The authors declare no conflict of interest.

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