Purification and Transplantation of Myogenic Progenitor Cell Derived Exosomes to Improve Cardiac Function in Duchenne Muscular Dystrophic Mice

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Abstract

Duchenne Muscular Dystrophy (DMD) is an X-linked recessive genetic disease caused by a lack of functional dystrophin protein. The disease cannot be cured, and as the disease progresses, the patient develops symptoms of dilated cardiomyopathy, arrhythmia, and congestive heart failure. The DMD^{MDX} mutant mice do not express dystrophin, and are commonly used as a mouse model of DMD. In our recent study, we observed that intramyocardial injection of wide type (WT)-myogenic progenitor cells-derived exosomes (MPC-Exo) transiently restored the expression of dystrophin in the myocardium of DMD^{MDX} mutant mice, which was associated with a transient improvement in cardiac function suggesting that WT-MPC-Exo may provide an option to relieve the cardiac symptoms of DMD. This article describes the technique of MPC-Exo purification and transplantation into hearts of DMD^{MDX} mutant mice.

Keywords

Genetics; Issue 146; Exosome; Duchenne muscular dystrophy; Myogenic progenitor cell; sequential ultracentrifugation; Exosome Transplantation; echocardiography; heart function

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive, progressive neuromuscular disease caused by a mutation in DMD gene and the loss of functional dystrophin¹. Dystrophin is expressed primarily in skeletal muscle and myocardium, and is less expressed in smooth muscle, endocrine glands and neurons^{2,3}. DMD is the most common type of muscular dystrophy with an incidence of one per 3,500 to 5,000 newborn boys worldwide^{4,5}. Individuals typically develop progressive muscle necrosis, loss of independent walking by

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Disclosures

All authors declares that they have no conflicts of interest.

Video Link

The video component of this article can be found at <https://www.jove.com/video/59320/>

early adolescence, and death in the second to third decades of their lives due to heart failure and respiratory failure⁶.

Dilated cardiomyopathy, arrhythmias and congestive heart failure are common cardiovascular manifestations of DMD^{7,8}. The disease can't be cured, supportive treatment may improve symptoms or delay the progression of heart failure, but it is very difficult to improve the heart function^{9,10}.

Similar to DMD patients, X-linked muscular dystrophy (MDX) mice are deficient in dystrophin protein and present symptoms of cardiomyopathy¹¹, and are therefore widely used in DMD associated cardiomyopathy research. In order to restore dystrophin in affected muscles, allogeneic stem cell therapy has proven to be an effective treatment for DMD^{12,13,14}. Exosomes, 30-150 nm membrane vesicles secreted by various cell types play a key role in cell-to-cell communication through genetic material transport, such as messenger RNA (mRNA) and non-coding RNAs^{15,16,17,18,19,20,21}.

Our previous studies have shown that exosomes derived from myogenic progenitor cells (MPC), such as C2C12 cell line, can transfer dystrophin mRNA to host cardiomyocytes after direct cardiac injection²², indicating that allogeneic delivery of MPC-derived exosomes (MPC-Exo) can transiently restore DMD gene expression in MDX mice. This article focuses on MPC-Exo purification and transplantation techniques.

Protocol

Animals were handled according to approved protocols and animal welfare regulations of the Institutional Animal Care and Use Committee of the Medical College of Georgia at Augusta University.

1. Isolation and Purification of MPC-derived Exosomes

1. Seed 5×10^6 C2C12 cells in a 15 cm cell culture dish with 20 mL complete Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin G and 100 μ g/mL streptomycin. Incubate at 37 °C and 50% CO₂.
2. Prepare exosome-depleted medium by ultracentrifugation of FBS at 100,000 x *g* for 18 h at 4 °C using a swinging bucket rotor. Discard the pellet.
3. Replace the complete DMEM with exosome-depleted medium when monolayer cells reach 80% confluence in the culture dish.
4. Use a transfer pipette to collect the supernatant from the cell culture dish every 48 h for a total of three times. Collect the supernatant in 50 mL centrifuge tubes, and centrifuge at 150 x *g* for 10 min to remove the remaining cells.
5. Filter the supernatant through a 0.22 μ m filter to eliminate cell debris. Ultracentrifuge the filtered medium in ultra-clear tubes using the swinging bucket rotor at 100,000 x *g* for 120 min at 4 °C to precipitate exosomes.

6. Resuspend the exosome-containing pellet in phosphate buffered saline (PBS) and fill the entire ultra-dear tube with PBS. Perform ultracentrifugation of this suspension at 100,000 x *g* for 120 min at 4 °C to eliminate contaminating proteins.
7. Discard the supernatant and resuspend the exosome pellet in 100 µL PBS. Store at –80 °C for future use.
8. For electron microscopy analysis, place 3 µL of exosomal suspension on a 200 mesh formvar-coated copper electron microscope grid for 5 min at room temperature. Continue with standard uranyl acetate staining²³. Examine the semi-dry grid by transmission electron microscopy.

2. Intramyocardial Exosome Delivery

NOTE: We used six mice in each group.

1. Anesthetize DBA/2J-DMD^{MDX} mice (male; age > 10 months; body weight (BW) > 18 g) with intraperitoneal (i.p.) injection of 100 mg/kg BW of ketamine combined with 10 mg/kg BW of xylazine, and confirm the depth of anesthesia by toe pinch.
2. Fix each mouse in supine position on a surgical platform by using tape to secure each limb and place a 3-0 suture horizontally below the upper teeth to hold the upper jaw in place.
3. Apply the depilatory cream to the left side of the mouse chest to remove the fur from the skin.
4. Perform endotracheal intubation via oral cavity using a 24 G catheter, and ventilate the mouse with room air at a rate of 195 breaths/min using a rodent ventilator (see the Table of Materials).
5. Disinfect the skin with 75% alcohol and 10% povidone iodine.
6. Make a 10–15 mm oblique incision from the left sternal edge to the left armpit, cut the pectoralis major and pectoralis minor by a scissor, then make a left thoracotomy through the fourth intercostal space. Gently insert the retractor bands to spread the thoracic cavity to a width of 10 mm. Take care to avoid damage to the left lung.
7. Use two straight tweezers to remove the pericardium, pull them apart, and place them behind the retractor tips to expose the heart. Inject MPC-Exo (50 µg in 30 µL PBS) or 30 µL PBS (as a control) intramyocardially into the anterior wall of the left ventricle using a 31 G insulin needle at one site.
8. Use 6-0 nylon sutures to close the thoracic cavity, pectoralis muscles and skin in sequence.
9. Recover mice. Once rhythmic, spontaneous breathing is present, remove the mice from the ventilator, and extubate. Observe and record the mice condition after surgery.

3. Echocardiography

NOTE: A single observer blinded to the experimental groups performs echocardiography and data analysis.

1. Two days after PBS/exosome transplantation, anesthetize the mice with 1–2% isoflurane.
2. Fix a mouse in supine position using tape, and apply the preheated acoustic gel on the left chest area.
3. Assess left ventricular function by echocardiography as previously described^{16,24}.
 - a. Obtain the parasternal long axis view of the left ventricle (LV) in two dimensions (B mode), and then rotate the ultrasound probe 90° to obtain a LV short-axis view at papillary muscle level.
 - b. Record M-mode echocardiographic images and measure left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic volume (LVESD), left ventricular end-diastolic volume (LVEDV), and left ventricular end-systolic volume (LVESV).

NOTE: Fractional shortening (FS%) = $[(LVEDD - LVESD) / LVEDD] \times 100$, and left ventricular ejection fraction (LVEF%) = $[(LVEDV - LVESV) / LVEDV] \times 100$.

4. Immunohistochemistry

1. Two days after PBS/exosome transplantation, anesthetize mice (see step 2.1) and cut the sternum open to expose the thoracic cavity. Dissect the inferior vena cava, and perfuse the heart through the left ventricle apex with 3 mL PBS, followed by 3 mL of 4% paraformaldehyde at room temperature, using a butterfly catheter with a 25 G needle attached to a 5 mL syringe.
2. Embed fixed hearts into paraffin, and cut into 5 µm thick sections.
3. Deparaffinize slides in xylene and rehydrate in ethanol solutions in following order: 100% xylene (3 min), 100% xylene (3 min), 100% ethanol (1 min), 100% ethanol (1 min), 95% ethanol (1 min), 80% ethanol (1 min), H₂O (1 min).
4. Fix tissue sections with 4% paraformaldehyde for 8 min at room temperature.
5. Immerse slides in sodium citrate (0.01 M, pH 6.0) and perform antigen retrieval using an antigen retriever.
6. Permeabilize sections for 1 h at room temperature with 1% polyethylene glycol tert-octylphenyl ether in PBS.
7. Block sections with 5% goat serum in PBS for 1 h at room temperature.
8. Incubate sections with diluted primary antibody (anti-dystrophin antibody) in PBS (1:100) overnight at 4 °C, then wash three times with PBS for 5 min.

9. Incubate sections with diluted secondary antibody (Alexa Fluor 488 conjugated goat anti-rabbit IgG) in PBS (1:400) for 45 min at room temperature.
10. Control autofluorescence using an autofluorescence quenching kit (see the Table of Materials).
11. Use mounting medium containing DAPI (see the Table of Materials) to mount the slides.
12. Observe slides under a confocal microscope.

Representative Results

A flow chart for isolating and purifying exosomes from C2C12 cells is shown in Figure 1A. To confirm the presence of exosomes, we performed transmission electron microscopy analysis. Transmission electron microscopy image (Figure 1B) shows the morphology of the bright and round shape vesicles of C2C12 derived exosomes. Western blot analysis confirmed the presence of exosome markers, including CD63 and TSG101 (Figure 1C).

We observed a translucent edema area after intramyocardial PBS/MPC-Exo injection into the anterior wall of the left ventricle of MDX mice (Figure 2), indicating the successful injection into the myocardium.

To determine whether cardiac MPC-Exo delivery restores dystrophin protein expression in MDX hearts, we performed immunofluorescent staining for dystrophin, and imaging with a confocal microscope. We observed partial restoration of dystrophin expression with membrane localization in some of cardiomyocytes (Figure 3A). To determine whether transplantation of MPC-Exo improves the cardiac function in MDX mice, we measured cardiac function by echocardiography 2 days after intramyocardial PBS / MPC-Exo delivery. As shown in Figure 3B, MPC-Exo treatment improved anterior wall movement compared with PBS, suggesting that MPC-Exo transplantation improved cardiac function in MDX mice.

Discussion

The method of isolating pure exosomes is essential for studying the function of exosomes. One of the common techniques for exosome isolation is polyethylene glycols (PEGs) mediated precipitation^{17,18,25}. Exosomes can be precipitated in PEGs, and pelleted by low-speed centrifugation. PEG-mediated purification is very convenient, low-cost, it does not need any advanced equipment, but there is concern about the purity of exosomes since other lipoproteins may be precipitated together and are difficult to remove. Ultrafiltration is a routine method for exosome separation, based upon the molecular weight and exclusion size limits²⁶. Ultrafiltration isolation is faster than ultracentrifugation based separation, but it can cause structural damage to large vesicles. The presence of various epitopes on the membrane of exosomes, such as CD9, CD63 and CD81²⁷, provides an alternative method of isolating exosomes by immunoaffinity interactions between these epitopes and antibodies bound to magnetic beads. Although immunoisolation has a high specificity²⁸, this technique has disadvantages of only isolating a subset of the total exosome population and may distort the

results of the experiment. Therefore, ultracentrifugation seems to be more suitable for this study on exosome function.

In this paper, we present a method for exosomes purification by sequential ultracentrifugation. After removing the remaining cells from the supernatant by centrifugation at 150 x *g*, the cell debris, apoptotic bodies and vesicles larger than 220 nm were removed by passing the supernatant through a 0.22 µm filter. Exosomes were then pelleted at 100,000 x *g* by initial ultracentrifugation. To remove possible protein contamination, we performed a second 100,000 x *g* ultracentrifugation after resuspending the exosome pellet in PBS. As per our experience, the advantage of sequential ultracentrifugation is the production of exosomes with high purity and cost-efficiency, however, it has disadvantage of low yield.

In order to allow the injected exosomes to cover most of the left ventricle and avoid leakage, we performed one intramyocardial injection using a 31 G insulin needle with the tip bent at about 20°. This technique is critical for the successful delivery of most exosomes into the myocardium and maximizes exposure of injected exosomes to host cardiomyocytes. In addition, we recommend injecting exosomes into the central region of the anterior wall of the left ventricle. After the injection is completed, we usually hold the needle at the injection site for 1 min to prevent liquid leakage. Successful injections were confirmed by the presence of a translucent edema around the injection site.

In our study, we found that allogeneic MPC-Exo transplantation can transiently restore dystrophin expression in heart and improve cardiac function in MDX mice²², which may provide new strategies for symptom relief in DMD patients. Since we observed recovered dystrophin protein expression in MDX mouse hearts, we assume that this is the mechanism for improved heart function, however, we cannot exclude other mechanisms, such as anti-inflammation; a recent report demonstrated that mesenchymal stem cell-derived exosomes improve the microenvironment of infarcted myocardium contributing to angiogenesis and anti-inflammation²⁹, moreover, Aminzadeh et al.³⁰ recently reported that cardiosphere-derived cells (CDCs) and their exosomes could transiently restore the expression of full length dystrophin in DMD mice. Considering that DMD is a systemic disease involving multiple organs, the local myocardial delivery of exosome is not suitable for treating respiratory failure due to diaphragm myopathy. Thus, systemic administration of exosomes, such as intravenous injection, has therapeutic potential, however, the major challenge is to develop an effective strategy for targeting exosomes to multiple muscle tissues. More importantly, exosome treatment has only a transient effect on partially restoring dystrophin expression, and improving heart function. More effective, longterm DMD treatment is needed.

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Figure 1: Exosome purification and characterization by electron microscopy.

(A) The diagram shows each step of isolation and purification of MPC-Exo. (B) The image shows round and cup-shaped vesicles under electron microscopy. Scale bar = 100 nm. (C) Western blot analysis confirmed the presence of exosome markers CD63 and TSG101.

Figure 2: Intramyocardial injection of PBS or MPC-Exo.

A translucent edema was observed in the left ventricular anterior wall after intramyocardial PBS/exosome delivery. (A) PBS injection. (B) MPC-Exo injection.

Figure 3: Intramyocardial delivery of MPC-Exo partially recovers dystrophin expression in MDX hearts, and improves heart function measured by echocardiography.
(A) Confocal immunofluorescent staining the heart sections of MDX mice 2 days after intramyocardial PBS/ MPC-Exo delivery. Blue = DAPI, Green = anti-dystrophin antibody.
(B) Echocardiographic measurements of cardiac function after 2 days of PBS/ MPC-Exo treatment.

Materials

Name	Company	Catalog Number	Comments
0.22 µm Filter	Fisherbrand	09-720-004	
15 cm Cell Culture Dish	Thermo Fisher Scientific	157150	
24 G catheter	TERUMO	SR-OX2419CA	
31 G insulin needle	BD	328291	
4% paraformaldehyde	Affymetrix	AAJ19943K2	
50 mL Centrifuge Tubes	Thermo Fisher Scientific	339652	
6-0 suture	Pro Advantage by NDC	P420697	
Alexa Fluor 488 goat anti-rabbit IgG	Thermo Fisher Scientific	A-11008	
Antibiotic Antimycotic Solution	Corning	30-004-CI	
Anti-Dystrophin antibody	Abcam	ab15277	
Antigen retriever	Aptum Biologics	R2100-US	Antigen recovery
Autofluorescence Quenching Kit	Vector Laboratories	SP-8400	
C2C12 cell line	ATCC	CRL-1772	
Centrifuge	Unico	C8606	
Change-A-Tip High Temp Cauteries	Bovie Medical Corporation	HIT	
Confocal microscopy	Zeiss	Zeiss 780 Upright Confocal	
DBA/2J-mdx mice	The Jackson Laboratory	013141	
DMEM	Corning	10-013-CM	
Fetal Bovine Serum (FBS)	Corning	35-011-CV	
Goat serum	MP Biomedicals, LLC	191356	
Isoflurane	Patterson Veterinary	07-893-1389	
Ketamine	Henry Schein	056344	
Mounting Medium with DAPI	Vector Laboratories	H-1500	
Mouse Retractor Set	Kent Scientific	SURGI-5001	
Polyethylene glycol tert-octylphenyl ether	Fisher Scientific	BP151-100	
Rodent ventilator	Harvard Apparatus	55-7066	
SW-28 Ti rotor	Beckman	342207	
The Vevo 2100 Imaging Platform	FUJIFILM VisualSonics	Vevo 2100	Ultrasound System
Ultracentrifuge	Beckman	365672	
Ultra-Clear Tubes	Beckman	344058	
Xylazine (XylaMed)	Bimeda-MTC Animal Health Inc.	1XYL003 8XYL006	