Journal of Visualized Experiments

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

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59320R1
Full Title:	Purification and Transplantation of Myogenic Progenitor Cell-Derived Exosomes to Improve Cardiac Function in Duchenne Muscular Dystrophic Mice
Keywords:	Exosome, Duchenne muscular dystrophy, Myogenic progenitor cell, sequential ultracentrifugation, Exosome Transplantation, echocardiography, heart function
Corresponding Author:	Yaoliang Tang Augusta University Augusta, GA UNITED STATES
Corresponding Author's Institution:	Augusta University
Corresponding Author E-Mail:	YAOTANG@augusta.edu
Order of Authors:	Yaoliang Tang
	Xuan Su
	Yan Shen
	Yue Jin
	Meng Jiang
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Augusta, GA, USA

TITLE:

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

AUTHORS & AFFILIATIONS:

Xuan Su[1,2]*, Yan Shen[1*], Yue Jin[1,2]*, Meng Jiang[2], Yaoliang Tang[1]

[1]Vascular Biology Center, Medical College of Georgia, Augusta University, Augusta, GA, USA
[2]Renji Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China 200127
*Equal Contribution to this work

E-mail Addresses of Co-Authors:
Xuan Su (xsu@augusta.edu)
Yan Shen (yashen@augusta.edu)
Yue Jin (yujin@augusta.edu)

Corresponding Author:
Yaoliang Tang (yaotang@augusta.edu)
Meng Jiang (jiangmeng0919@163.com)

KEYWORDS: Exosome, Duchenne muscular dystrophy, Myogenic progenitor cell, sequential ultracentrifugation, Exosome Transplantation, echocardiography, heart function

SUMMARY:
Here, we present a protocol to transiently improve cardiac function in Duchenne muscular dystrophy mice by transplanting exosomes derived from normal myogenic progenitor cells.

ABSTRACT:
Duchene 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.

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 dystrophin[1]. 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 3500 to 5000 newborn boys worldwide[4,5]. Individuals typically develop progressive muscle necrosis, loss of independent walking by early adolescence, and death in the second to third decades of their lives due to heart failure and respiratory failure[6].

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 cardiomyopathy[11], 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[12-14]. 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[15-21] .

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 injection[22], 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

1.1) Seed 5 x 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 µg/mL streptomycin. Incubate at 37 °C and 5% CO_2.

1.2) 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.

1.3) Replace the complete DMEM with exosome-depleted medium when monolayer cells reach 80% confluence in the culture dish.

1.4) 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.

1.5) Filter the supernatant through a 0.22 µm 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.

1.6) Resuspend the exosome-containing pellet in phosphate buffered saline (PBS) and fill the entire ultra-clear tube with PBS. Perform ultracentrifugation of this suspension at 100,000 x *g* for 120 min at 4 °C to eliminate contaminating proteins.

1.7) Discard the supernatant and resuspend the exosome pellet in 100 µL PBS. Store at -80 °C for future use.

1.8) 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 staining[23]. Examine the semi-dry grid by transmission electron microscopy.

2. Intramyocardial Exosome Delivery

NOTE: We used six mice in each group.

2.1) 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.

2.2) 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.

2.3) Apply the depilatory cream to the left side of the mouse chest to remove the fur from the skin.

2.4) Perform endotracheal intubation via oral cavity using a 24 G catheter, and ventilate the mouse with room air at a rate of 195 breaths per minute using a rodent ventilator (see the **Table of Materials**).

2.5) Disinfect the skin with 75% alcohol and 10% povidone iodine.

2.6) 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.

2.7) 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.

2.7) Use 6-0 nylon sutures to close the thoracic cavity, pectoralis muscles and skin in sequence.

2.8) 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.

3.1) Two days after PBS / exosome transplantation, anesthetize the mice with 1–2% isoflurane.

3.2) Fix a mouse in supine position using tape, and apply the preheated acoustic gel on the left chest area.

3.3) Assess left ventricular function by echocardiography as previously described[16,24].

3.3.1) 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.

3.3.2) 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] × 100, and left ventricular ejection fraction (LVEF%) = [(LVEDV − LVESV) / LVEDV] × 100.

4. Immunohistochemistry

4.1) 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.

4.2) Embed fixed hearts into paraffin, and cut into 5 µm thick sections.

4.3) 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), H_2O (1 min).

4.4) Fix tissue sections with 4% paraformaldehyde for 8 min at room temperature.

4.5) Immerse slides in sodium citrate (0.01 M, pH 6.0) and perform antigen retrieval using an antigen retriever.

4.6) Permeabilize sections for 1 h at room temperature with 1% polyethylene glycol tert-octylphenyl ether in PBS.

4.7) Block sections with 5% goat serum in PBS for 1 h at room temperature.

4.8) 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.

4.9) Incubate sections with diluted secondary antibody (Alexa Fluor 488 conjugated goat anti-rabbit IgG) in PBS (1:400) for 45 min at room temperature.

4.10) Control autofluorescence using an autofluorescence quenching kit (see the **Table of Materials**).

4.11) Use mounting medium containing DAPI (see the **Table of Materials**) to mount the slides.

4.12) 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.

Figure legends:

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.

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 limits[26]. 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 CD81[27], provides an alternative method of isolating exosomes by immunoaffinity interactions between these epitopes and antibodies bound to magnetic beads. Although immunoisolation has a high specificity[28], 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 mice[22], 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-inflammation[29], 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, long-term DMD treatment is needed.

ACKNOWLEDGMENTS:

Tang were partially supported by the American Heart Association: GRNT31430008, NIH-AR070029, NIH-HL086555, NIH-HL134354.

DISCLOSURES:

All authors declares that they have no conflicts of interest.

REFERENCES:

1 Yiu, E. M., Kornberg, A. J. Duchenne muscular dystrophy. *Journal of Paediatrics and Child Health.* **51** (8), 759-764, (2015).

2 Nudel, U., et al. Duchenne muscular dystrophy gene product is not identical in muscle and brain. *Nature.* **337** (6202), 76-78, (1989).

3 Rae, M. G., O'Malley, D. Cognitive dysfunction in Duchenne muscular dystrophy: a possible role for neuromodulatory immune molecules. *Journal of Neurophysiology.* **116** (3), 1304-1315, (2016).

4 Mah, J. K., et al. A systematic review and meta-analysis on the epidemiology of Duchenne and Becker muscular dystrophy. *Neuromuscular Disorders.* **24** (6), 482-491, (2014).

5 D'Amario, D., et al. A current approach to heart failure in Duchenne muscular dystrophy. *Heart.* **103** (22), 1770-1779, (2017).

6 Koeks, Z., et al. Clinical Outcomes in Duchenne Muscular Dystrophy: A Study of 5345 Patients from the TREAT-NMD DMD Global Database.Journal of Neuromuscular Diseases. **4** (4), 293-306, (2017).

7 Kamdar, F., Garry, D. J. Dystrophin-Deficient Cardiomyopathy. *Journal of the American College of Cardiology.* **67** (21), 2533-2546, (2016).

8 Wang, Z., et al. Regenerative Therapy for Cardiomyopathies. *Journal of Cardiovascular Translational Research.* 10.1007/s12265-018-9807-z, (2018).

9 Fayssoil, A., Nardi, O., Orlikowski, D., Annane, D. Cardiomyopathy in Duchenne muscular dystrophy: pathogenesis and therapeutics. *Heart Failure Reviews.* **15** (1), 103-107, (2010).

10 Hagan, M., Ashraf, M., Kim, I. M., Weintraub, N. L., Tang, Y. Effective regeneration of dystrophic muscle using autologous iPSC-derived progenitors with CRISPR-Cas9 mediated precise correction. *Medical Hypotheses.* **110** 97-100, (2018).

11 Quinlan, J. G., et al. Evolution of the mdx mouse cardiomyopathy: physiological and morphological findings. *Neuromuscular Disorders.* **14** (8-9), 491-496, (2004).

12 Siemionow, M. et al. Creation of Dystrophin Expressing Chimeric Cells of Myoblast Origin as a Novel Stem Cell Based Therapy for Duchenne Muscular Dystrophy. *Stem Cell Reviews and Reports.* **14** (2), 189-199, (2018).

13 Sienkiewicz, D., Kulak, W., Okurowska-Zawada, B., Paszko-Patej, G., Kawnik, K. Duchenne muscular dystrophy: current cell therapies. *Therapeutic Advances in Neurological Disorders.* **8** (4), 166-177, (2015).

14 Zhang, Y., et al. Long-term engraftment of myogenic progenitors from adipose-derived stem cells and muscle regeneration in dystrophic mice. *Human Molecular Genetics.* **24** (21), 6029-6040, (2015).

15 Ju, C., et al. Transplantation of Cardiac Mesenchymal Stem Cell-Derived Exosomes for Angiogenesis. *Journal of Cardiovascular Translational Research.* **11** (5), 429-437, (2018).

16 Ju, C., et al. Transplantation of Cardiac Mesenchymal Stem Cell-Derived Exosomes Promotes Repair in Ischemic Myocardium. *Journal of Cardiovascular Translational Research.* **11** (5), 420-428, (2018).

17 Ruan, X. F., et al. Suxiao Jiuxin pill promotes exosome secretion from mouse cardiac mesenchymal stem cells in vitro. *Acta Pharmacologica Sinica.* **39** (4), 569-578, (2018).

18 Ruan, X. F., et al. Exosomes from Suxiao Jiuxin pill-treated cardiac mesenchymal stem cells decrease H3K27 demethylase UTX expression in mouse cardiomyocytes in vitro. *Acta Pharmacologica Sinica.* **39** (4), 579-586, (2018).

19 Chen, Y., Tang, Y., Fan, G. C., Duan, D. D. Extracellular vesicles as novel biomarkers and pharmaceutic targets of diseases. *Acta Pharmacologica Sinica.* **39** (4), 499-500, (2018).

20 Chen, Y., Tang, Y., Long, W., Zhang, C. Stem Cell-Released Microvesicles and Exosomes as Novel Biomarkers and Treatments of Diseases. *Stem Cells International.* **2016** 2417268, (2016).

21 Murphy, C., et al. Emerging role of extracellular vesicles in musculoskeletal diseases. *Molecular Aspects of Medicine.* **60** 123-128, (2018).

22 Su, X., et al. Exosome-Derived Dystrophin from Allograft Myogenic Progenitors Improves Cardiac Function in Duchenne Muscular Dystrophic Mice. *Journal of Cardiovascular Translational Research.* 10.1007/s12265-018-9826-9, (2018).

23 Hu, G., et al. Exosome-mediated shuttling of microRNA-29 regulates HIV Tat and morphine-mediated neuronal dysfunction. *Cell Death & Disease.* **3** e381, (2012).

24 Bayoumi, A. S., et al. A carvedilol-responsive microRNA, miR-125b-5p protects the heart from acute myocardial infarction by repressing pro-apoptotic bak1 and klf13 in cardiomyocytes. *Journal of Molecular and Cellular Cardiology.* **114** 72-82, (2018).
25 Wang, Y., et al. Exosomes/microvesicles from induced pluripotent stem cells deliver cardioprotective miRNAs and prevent cardiomyocyte apoptosis in the ischemic myocardium. *International Journal of Cardiology.* **192** 61-69, (2015).
26 Cheruvanky, A., et al. Rapid isolation of urinary exosomal biomarkers using a nanomembrane ultrafiltration concentrator. *American Journal of Physiology-Renal Physiology.* **292** (5), F1657-1661, (2007).
27 Oksvold, M. P., Neurauter, A., Pedersen, K. W. Magnetic bead-based isolation of exosomes. *Methods in Molecular Biology.* **1218** 465-481, (2015).
28 Pedersen, K. W., Kierulf, B., Neurauter, A. Specific and Generic Isolation of Extracellular Vesicles with Magnetic Beads. *Methods in Molecular Biology.* **1660** 65-87, (2017).
29 Teng, X., et al. Mesenchymal Stem Cell-Derived Exosomes Improve the Microenvironment of Infarcted Myocardium Contributing to Angiogenesis and Anti-Inflammation. *Cellular Physiology and Biochemistry.* **37** (6), 2415-2424, (2015).
30 Aminzadeh, M. A., et al. Exosome-Mediated Benefits of Cell Therapy in Mouse and Human Models of Duchenne Muscular Dystrophy. *Stem Cell Reports.* **10** (3), 942-955, (2018).

Figure 1

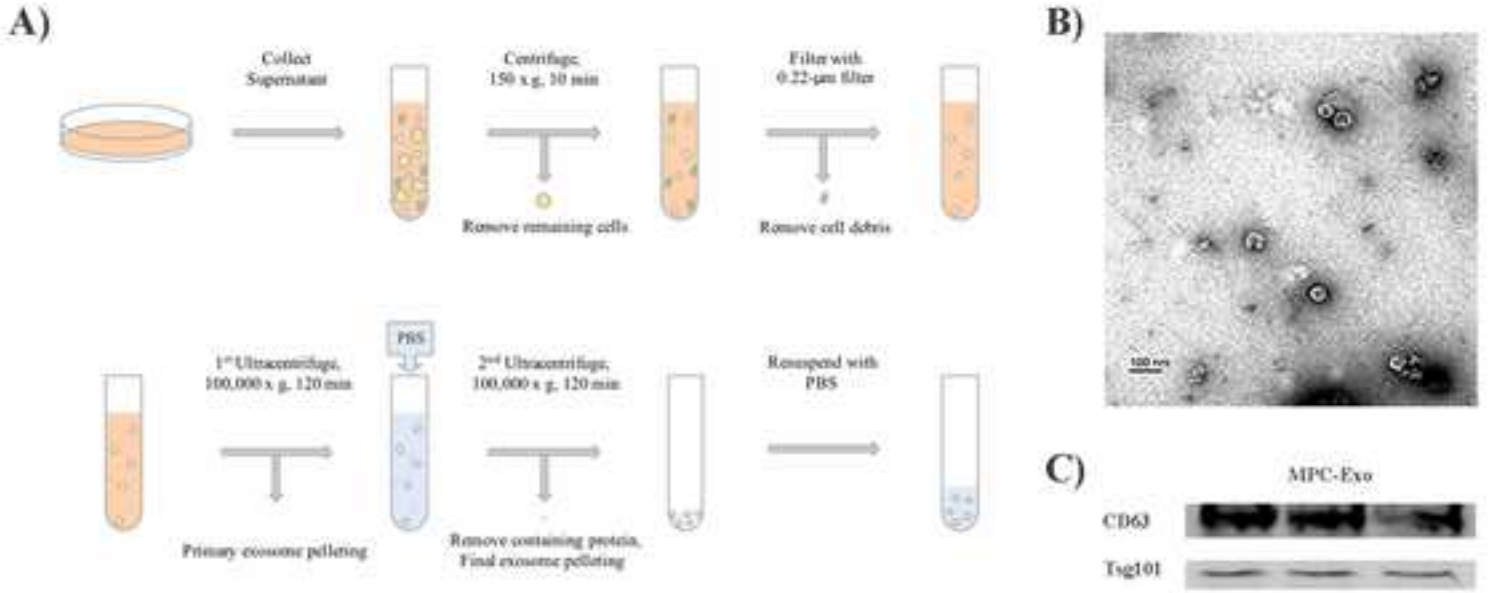


Figure 2

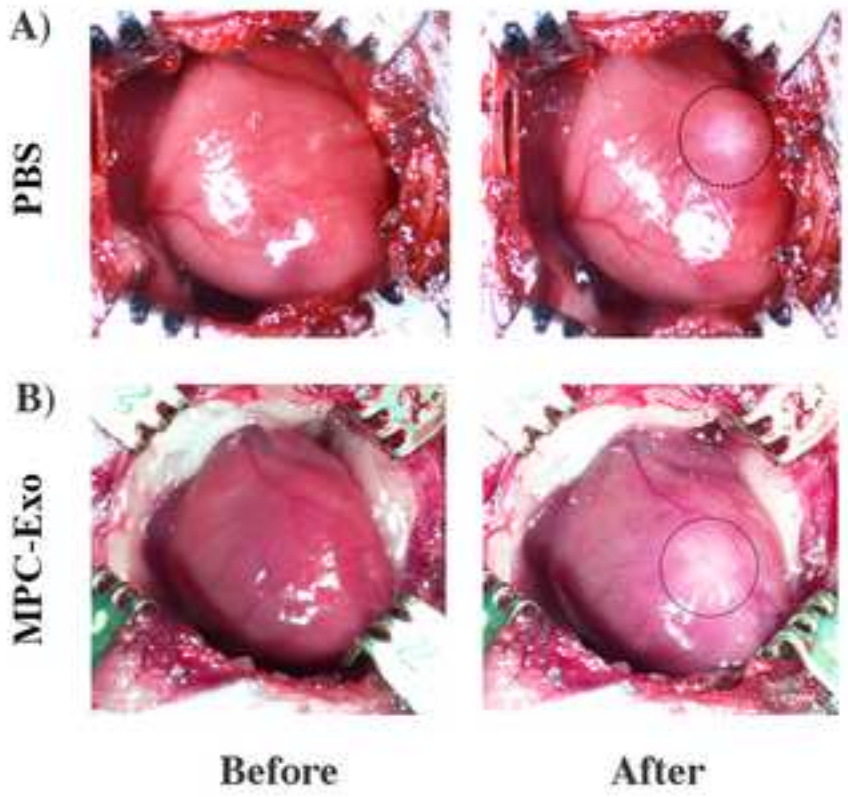
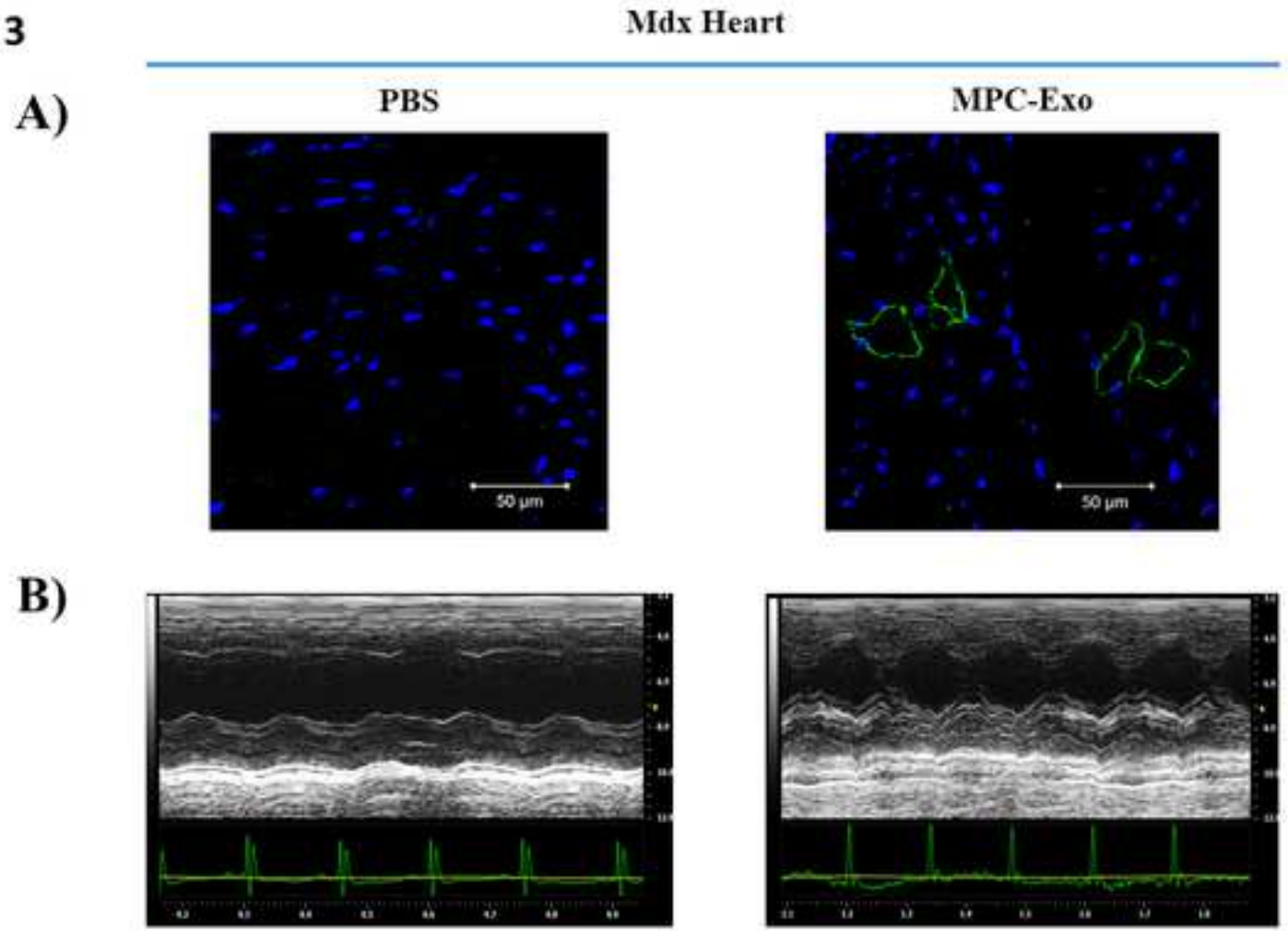


Figure 3



Name of Material/ Equipment
0.22-µm Filter
15-cm Cell Culture Dish
24-gauge catheter
31-gauge insulin needle
4% paraformaldehyde
50 mL Centrifuge Tubes
6-0 suture
Alexa Fluor 488 goat anti-rabbit IgG
Antibiotic Antimycotic Solution
Anti-Dystrophin antibody
Antigen retriever
Autofluorescence Quenching Kit
C2C12 cell line
Centrifuge
Change-A-Tip High Temp Cauteries
Confocal microscopy
DBA/2J-mdx mice
DMEM
Fetal Bovine Serum (FBS)
Goat serum
Isoflurane
Ketamine
Mounting Medium with DAPI
Mouse Retractor Set
Polyethylene glycol tert-octylphenyl ether
Rodent ventilator
SW-28 Ti rotor
The Vevo 2100 Imaging Platform
Ultracentrifuge
Ultra-Clear Tubes
Xylazine (XylaMed)

Company	Catalog Number	Comments/Description
Fisherbrand	09-720-004	
Thermo Fisher Scientific	157150	
TERUMO	SR-OX2419CA	
BD	328291	
Affymetrix	AAJ19943K2	
Thermo Fisher Scientific	339652	
Pro Advantage by NDC	P420697	
Thermo Fisher Scientific	A-11008	
Corning	30-004-CI	
Abcam	ab15277	
Aptum Biologics	R2100-US	Antigen recovery
Vector Laboratories	SP-8400	
ATCC	CRL-1772	
Unico	C8606	
Bovie Medical Corporation	HIT	
Zeiss	Zeiss 780 Upright Confocal	
The Jackson Laboratory	013141	
Corning	10-013-CM	
Corning	35-011-CV	
MP Biomedicals, LLC	191356	
Patterson Veterinary	07-893-1389	
Henry Schein	056344	
Vector Laboratories	H-1500	
Kent Scientific	SURGI-5001	
Fisher Scientific	BP151-100	
Harvard Apparatus	55-7066	
Beckman	342207	
FUJIFILM VisualSonics	Vevo 2100	Ultrasound System
Beckman	365672	
Beckman	344058	
Bimeda-MTC Animal Health Inc.	1XYL003 8XYL006	

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

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

Author(s): Xuan Su, Yan Shen, Yue Jin, Meng Jiang, Yaoliang Tang

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: [X] Standard Access [ ] Open Access

Item 2 (check one box):

[X] The Author is NOT a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	Yaoliang Tang
Department:	Vascular Biology Center
Institution:	Medical College of Georgia
Article Title:	Purification and Transplantation of Myogenic Progenitor Cell-Derived Exosomes to Improve Cardiac Function in Duchenne Muscular Dystrophic Mice
Signature:	
Date:	10/29/2018

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

   Answer: We did proofread.

2. Please provide an email address for each author.

   Answer: E-mail: Xuan Su, xsu@augusta.edu; Yan Shen, yashen@augusta.edu; Yue Jin, yujin@augusta.edu; Meng Jiang, jiangmeng0919@163.com; Yaoliang Tang, yaotang@augusta.edu.

3. Keywords: Please provide at least 6 keywords or phrases.

   Answer: Keywords: Exosome, Duchenne muscular dystrophy, Myogenic progenitor cell, sequential ultracentrifugation, Exosome Transplantation, echocardiography, heart function

4. Please add a Summary section before the Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to ..."

   Answer: We add SUMMARY: Here, we present a protocol to transiently improve cardiac function in Duchenne muscular dystrophy mice by transplanting exosomes derived from normal myogenic progenitor cells.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (TM), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Patterson Veterinary, Triton X-100, VECTASHIELD HardSet, etc.

   Answer: We have removed these commercial languages.

6. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

   Answer: We removed the personal pronous.

7. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

   Answer: We move the discussion about the protocol to the Discussion.

8. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

9. 1.4: Please describe how to collect the supernatant and where it is collected from.

Answer: Use a transfer pipette to collect the supernatant from the cell culture dish.

10. 1.8, 1.10: What volume of PBS is used?

Answer: 1.8 Fill up the entire ultra-clear tube.

1.10 100 μL.

11. 1.9: What is centrifuged? The suspension from step 1.8?

Answer: Yes, the suspension from step 1.8.

12. 2.1: Please mention how proper anesthetization is confirmed.

Answer: confirm the depth of anesthesia by toe pinch

13. 2.6: Is PBS served as control? Please specify.

Answer: PBS was used as a control.

14. 2.7: What is used to close the thoracic cavity, pectoralis muscles and skin?

Answer: 6-0 suture.

15. 3.1: Please specify the concentration of isoflurane.

Answer: We set isoflurane at 1-2% to control heart rate at above 400rpm.

16. 4.1: Please describe how to harvest mouse hearts and specify the temperature at which the heart was fixed.

Answer: We add following detail: anesthetize mice (see 2.1) and incise the skin from pelvis to chin, cut the sternum to open the chest. dissect the inferior vena cava, and perfuse the heart with 3ml PBS followed by 3ml 4% paraformaldehyde (room temperature), using a butterfly catheter with 25g needle attached to a 5 ml syringe with the tip positioned at apex of left ventricle.

17. 4.3: Please specify the concentrations of ethanol solutions used and the time for each treatment.

Answer: we add detail below: 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).

18. Please include single-line spaces between all paragraphs, headings, steps, etc.

Answer: We change to single-line spaces.

19. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Answer: We highlight 1.5 pages of protocol for the video.

20. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

Answer: We highlight the steps.

21. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Answer: We include all relevant details.

22. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

Answer: The discussion of critical steps within the protocol are there.

23. References: Please do not abbreviate journal titles.

Answer: we change the styles of journal titles.

24. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

Answer: We will upload each figure individually.

25. Please include a title for each figure.

Answer: We add title for each figure.

26. Table of Materials: Please remove trademark (TM) and registered (®) symbols and sort the items in alphabetical order according to the name of material/equipment.

Answer: We remove the trademark and registered symbol, and sorted the items.

Reviewers' comments:

Reviewer #1:

This is a well-written paper reporting.

Answer: We thank the reviewer for his/her positive comments.

Reviewer #2:

Manuscript Summary:

The paper describes a technique for isolating exosomes from myogenic progenitor cells and their intramyocardial injection in a mouse model of Duchenne muscular dystrophy. Results were assessed two days after exosome injections and show expression of dystrophin gene expression and improved cardiac function, as assessed by echocardiography.

Major Concerns:

1- The technique used for exosome purification is based on a series of untracentrifugations. As such, it does not provide any novel information in that this technique is routinely used for this purpose. Thus, claiming that the paper reports "an improved method for exosome purification" is not really true. Furthermore, in the discussion, the assumption that ultracentrifugation might be the most suitable method is questionable and biased by the inappropriate critiques of the other methods; thus, ultrafiltration is reported to possibly cause structural damage to "large vesicles", which may not be an issue if the target is to isolate exosomes and no mention is made of tangential filtration which is increasingly recommended.

Answer: Thanks to the reviewer's comments, we do not criticize other methods of purifying exosomes. We also routinely use PEG based protocol to purify exosomes and microvesicles. According to our experience, the advantage of sequential ultracentrifugation is the production of exosome with high purity and cost-efficiency, however, it has disadvantage of the low yield.

2- The characterization of exosomes if not fully convincing. Although Figure 1B shows a round-cup morphology of the isolated particles, the data would have been more meaningful if electron microscopy had been combined with immunostaining against typical markers like CD63 or CD81. Representative Nanoparticle Tracking Analysis spectra should also have been provided to give information on the number and size distribution of the isolated particles.

Answer: Thanks for the reviewer's comments. The detailed characterization of exosome, including nanoparticle tracking analysis, was published (doi: 10.1007/s12265-018-9826-9), we added Western blot data, which confirmed the presence of exosome markers, including CD63 and TSG101 (Fig. 1C).

3- In the animal study, no data are given regarding the number of mice in each group, the use or not of randomization and blind assessment of outcomes, the nature of the control medium which was presumably injected. No mention is made either on the limitations of the mdx model for duplicating the patterns seen in Duchenne myopathy.

Answer: Thanks for the reviewer's comments, we had 6 mice in each group, see detail in our published paper (doi: 10.1007/s12265-018-9826-9). We agree with the review that there are limitations of using mdx mice to duplicating the symptoms in Duchenne myopathy. We used

DBA/2J-DmdMDX mice, which is a superior Duchenne muscular dystrophy model as it better recapitulates several of the human characteristics of DMD pathology (Jackson laboratory).

4- While the rapid internalization of exosomes in the target cells might account for the expression of dystrophin shown in Figure 3A, the functional data are more than questionable in view of the shortness of follow-up (2 days). Further doubts about the reality of the reported functional improvement come from the fact that neither posttransplantation nor baseline actual echocardiographic values are reported while it would be really surprising that function can improve so rapidly (2 days after treatment). Furthermore, the histological data are limited to an image of dystrophin expression without details about the number of hearts/slides in which this result was observed and without any information on other important histological patterns such as those in relation with inflammation and a possible immune response.

Answer: Thanks for reviewer's comments, we randomly selected 10 fields in each heart section, and assigned the field with dystrophin- positive cardiomyocytes as positive field, and compared the percentage of positive field in each heart section between PBS- and MPC-Exo-treated hearts of MDX mice ($n = 18$, $^*p < 0.05$). See our published paper (doi: 10.1007/s12265-018-9826-9). Since we observed recovered dystrophin protein expression in Mdx mouse hearts, we assume this is the mechanisms 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-Inflammation (doi: 10.1159/000438594), moreover, Aminzadeh MA et al recently reported that cardiosphere-derived cells (CDCs) and their exosomes could transiently restore the expression of full length dystrophin in DMD mice (doi: 10.1016/j.stemcr.2018.01.023).

Reviewer #3:

Manuscript Summary:

Overall, this manuscript is well written and it will be an useful method for the scientific community to purify the exosomes in different origins. This manuscript can be accepted in the present form.

Answer: We thank reviewer for this positive comment.

Reviewer #4:

Manuscript Summary:

This article describes the technique of MPC-Exo purification and transplantation into hearts of Dmdmdx mutant mice.

Major Concerns: None noted.

Minor Concerns:

In the heart image, cam Can the authors show the MPC exosomes are positive for CD9, CD63 and CD812?

Answer: Thanks for reviewer's comments, we did co-stain dystrophin with Tsg101, an exosome marker, in the Mdx hearts treated with MPC-Exo and PBS, however, we did not observe obvious difference of Tsg101 expression in cardiomyocytes with restored dystrophin expression in MPCExo-treated hearts in comparison with cardiomyocytes without dystrophin expression in PBS-treated hearts, see our publication (doi: 10.1007/s12265-018-9826-9).

Is there any quantitative data regarding the cardiac function results?

Answer: Thanks for reviewer's comments, the quantification of cardiac function was shown in our publication (doi: 10.1007/s12265-018-9826-9), Compared with PBS, MPC-Exo administration improved both left ventricular ejection fraction (EF 74.3 ± 2.5% vs 58.2 ± 3.4%, $p < 0.05$, $n = 6$) and fractional shortening (FS 42.5 ± 2.4% vs 30.0 ± 2.3%, $p < 0.05$, $n = 6$) in MDX mice.

Can the authors comment on the yield of exosomes from the cell culture using the ultracentrifugation method?

Answer: According to our experience, compared to PEG purification, the disadvantage of a series of ultracentrifugation is the low yield of exosomes.

Reviewer #5:

Manuscript Summary:

In the manuscript titled, "Purification and transplantation of myogenic progenitor cell-derived exosomes to improve cardiac function in Duchenne Muscular Dystrophic mice", Su X et al., describe a protocol for isolation, purification and transplantation of exosomes derived from myogenic cells in MDX mice. Overall, the protocol and corresponding results are well described. There are some concerns as highlighted that will improve the impact of the paper.

Major Concerns:

1. Authors must include methods and pertaining results to clarify whether the isolated exosome is indeed within the size range of 100-150. Moreover, include methods for immunoblot analysis of exosome phenotype.

Answer: Thanks for reviewer's comment, we measured the size of isolated exosomes using the Zeta analysis, see Figure 2 in our published paper (doi: 10.1007/s12265-018-9826-9).

2. Please include a picture of PBS only and PBS/exo hearts to clarify whether edema is due to exo/PBS or PBS only Minor Concerns:

Answer: Thanks for reviewer's comments, we added a picture of mouse heart before/after PBS injection (Figure 2A). And no significant difference was observed between PBS and Exo injections, indicating that the edema is a way of judging whether the myocardial injection was successful, regardless of the injected materials.

Section 2. Intramyocardial exosome delivery, sub section 2.6 mentions 50ug in 30ugPBS. Can the authors clarify what they mean? Also, please mention whether the exo/PBS were injected in a single injection site or multiple injections.

Answer: Thanks for comments, it is a typo, it has been corrected to "50ug in 30uL PBS". The exo/PBS were injected in a single injection site.

Reviewer #6:

Manuscript Summary:

This article introduces the techniques for exosome isolation from myogenic progenitor cells, purification, and transplantation into hearts of Duchenne muscular dystrophic mice. The title and abstract are appropriate for this methods articles. The steps listed in the procedure are clearly explained and would lead to the described outcome.

Minor Concerns:

a. Please check the formula for calculating LVEF.

Answer: We have checked the calculating LVEF.

b. Several typos:

83 1.11) '3µL of exosomal pellet' should be '3µl of exosomal suspension';

Answer: Thanks for pointing it out, we made correction.

100 2.6) '50µg in 30µg PBS' should be '50µg in 30µl PBS'.

Answer: Thanks for point it out, we made correction of this typo.

c. It would be better if the model and manufacture of Ultracentrifuge and Ultrasound System can be listed.

Answer: Thanks for this comment, we add this information to the list.