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Exosomes Derived dystrophin from Allograft Myogenic Progenitors Improve Cardiac Function in Duchenne Muscular Dystrophic Mice

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Abstract

Progressive cardiomyocyte loss in Duchenne muscular dystrophy (DMD) leads to cardiac fibrosis, cardiomyopathy and eventually heart failure. In the present study, we observed that myogenic progenitor cells (MPC) carry mRNA for the dystrophin gene. We tested whether cardiac function can be improved in DMD by allograft transplantation of MPC-derived exosomes (MPC-Exo) into the heart to restore dystrophin protein expression. Exo from C2C12 cells (an MPC cell line) or vehicle were delivered locally into the hearts of MDX mice. After 2 days of treatment, we observed that MPC-Exo restored dystrophin expression in the hearts of MDX mice, which correlated with improved myocardial function in dystrophin-deficient MDX mice hearts. In conclusion, this study demonstrated that allogeneic WT-MPC-Exo transplantation transiently restored dystrophin gene expression and improved cardiac function in MDX mice, suggesting that allogeneic exosomal delivery may serve as an alternative treatment for cardiomyopathy of DMD.

Keywords

Dystrophin; Exosome; Myogenic progenitor cells; Cardiomyopathy

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This article does not contain any studies with human participants performed by any of the authors.

Introduction:

Duchenne muscular dystrophy (DMD) is an X-linked genetic disorder caused by the absence of dystrophin, a gene that encodes a 427-kd protein that links the sarcomere and the extracellular matrix(1). Dystrophin protein functions to stabilize dystrophin-associated glycoprotein complex (DGC) during cyclic muscle contraction and relaxation(2). DMD patients have symptoms in early childhood, and gradually lose mobility during adolescence due to progressive muscle cell atrophy, which is replaced by fibrotic tissue, and patients typically die from heart or pulmonary failure in their third decade(3).

The most common cardiovascular manifestations of DMD are dilated cardiomyopathy, arrhythmias and congestive heart failure (4). Medical therapies, including corticosteroids, angiotensin-converting enzyme (ACE) inhibitors, beta-blockers, and antiarrhythmics can improve symptoms and potentially delay the progression of cardiac remodeling and heart failure(3); to date, however, there is no cure for cardiomyopathy associated with DMD.

Stem cell therapy is a promising approach to treat DMD(5). Progenitor cells release exosomes/microvesicles, which are extracellular nanovesicles mediating cell-cell communication by exchanging genetic material, including DNAs and RNAs(6). Aminzadeh MA et al (7) recently reported that cardiosphere-derived cells (CDCs) and their exosomes could transiently restore the expression of full-length dystrophin in DMD mice. It is unclear whether exosomes/microvesicles from muscle progenitor cells (MPC), such as the C2C12 cell line, can be used to restore cardiac dystrophin expression and improve heart function in the murine MDX model of DMD.

In this study, we evaluated the transient therapeutic efficacy of allogenic transplantation of myogenic progenitor cells-derived exosomes (MPC-Exo) into the hearts of MDX mice. Our results suggest that this approach can improve heart function in DMD mice, in association with increased expression of dystrophin in recipient hearts.

Methods:

Cell culture and exosome purification

The C2C12 cells (ATCC, Manassas, VA) were cultured and maintained in complete DMEM media containing 10% exosome depleted fetal bovine serum and 100 U/mL penicillin G, 100 µg/ml streptomycin. Exosomes released by the C2C12 cells were purified as described in the literature with minor modifications(8). Briefly, supernatant was centrifuged at 150 ($\times$ g) for 10min to eliminate cells, followed by filtration through 0.22µm filter to remove cell debris. The filtered media was ultracentrifuged using a SW-28 Ti rotor (Beckman Coulter Instruments) at 100,000 $\times$ g for 120 min at 4°C to pellet the exosomes. The pelleted exosomes were washed in phosphate-buffered saline (PBS) to eliminate contaminating proteins and re-ultracentrifuged at 100,000 $\times$ g for 120min at 4°C. The exosome pellets were resuspended in PBS overnight at 4°C, and stored at -80°C for later use.

Electron microscopy and Zeta analysis

For the transmission electron microscopy (TEM) morphology assessment, 3 μ L of exosome pellet was placed on formvar carbon-coated 200-mesh copper electron microscopy grids, incubated for 5 min at room temperature (RT), and then subjected to standard uranyl acetate staining(9). The grid was washed with three exchanges of PBS and allowed to semi-dry at room temperature before observation by transmission electron microscope (JEOL JEM 1230, Peabody, MA). Micrographs were used to quantify the diameter of exosomes. Exosome particle size was measured using nanoparticle tracking analysis (NTA) with ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) and corresponding software (ZetaView 8.02.28) as described previously(10-14). The ZetaView system was calibrated using 100 nm polystyrene particles.

Isolation and quantification of message RNA

Message RNAs (mRNA) are RNAs which transfer genetic information transcribed from DNA to a ribosome. Total RNA was extracted from Exo- or PBS-treated MDX hearts using RNeasy RT (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer's instructions. Complementary DNA (cDNA) is synthesized by a single stranded RNA, such as mRNA, in a reaction catalyzed by reverse transcriptase. cDNAs were synthesized from total RNA using RevertAid First Strand cDNA Synthesis kits (Thermo Scientific). The cDNA was used to perform quantitative PCR on CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using PowerUp SYBR Green Master Mix (ThermoFisher). Amplification was performed at 50°C for 2min, 95 °C for 2 min, followed by 50 cycles of 95 °C for 15 s, and 60 °C for 1 min with primers listed in **Table 1**.

Intramyocardial exosome delivery

Male MDX mice were anesthetized with intraperitoneal injection of 100 mg/kg ketamine combined with 10 mg/kg xylazine, intubated transorally with a 24-gauge tube and ventilated with room air using a Harvard rodent ventilator (Inspira Advanced Safety Ventilator Model 55-7058, Holliston, MA). The chest was opened via a lateral thoracotomy, and the heart was exposed through pericardiotomy. MPC-Exo (50ug in 30ul PBS) or PBS alone was injected into the left anterior wall (n = 6 per group). The chest was closed and the mice were allowed to recover. Animals were sacrificed at 2 days after exosome injection for tissue harvesting and histological assays. 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/Augusta University.

Assessment of heart function by echocardiography

Left ventricular performance was assessed by two-dimensional echocardiography using Vevo2100 Imaging System (Visualsonics) as previously described(15). Briefly, after the induction of general anesthesia with isoflurane (Patterson Veterinary), cardiac imaging was performed using 2-D and M-mode echocardiography. M-mode tracings were used to measure anterior and posterior wall movement at end diastole and end systole. Left ventricular internal diameter (LVID) was measured in either diastole (LVIDd) or systole (LVIDs). End diastolic volume (EDV) and end systolic volume (ESV) were also measured.

A single observer blinded to the experimental groups performed echocardiography and data analysis. Fractional shortening (FS) was calculated according to the following formula: $FS (\%) = [(LVIDd - LVIDs) / LVIDd] \times 100$. Ejection fraction (EF) was calculated as: $EF (\%) = [(EDV - ESV) / EDV] \times 100$.

Immunofluorescent staining

For heart staining to assess the dystrophin gene expression in MDX mice two days post PBS or Exo treatment, mouse hearts were harvested, embedded in OCT compound, snap-frozen, cut into 5- μ m sections, and immunostained with rabbit anti-dystrophin (1:100; abcam, Cambridge, MA) antibodies. Primary antibodies were resolved via secondary staining with goat anti-rabbit Alexa Fluor 488-conjugated (1:400, Life Technologies, Carlsbad, CA). Nuclei were counterstained with DAPI (Vector Laboratories, Burlingame, CA).

We also used a protocol for co-staining with mouse anti-Tsg101 (Exosome marker) with rabbit anti-dystrophin in mouse heart slices with modification (16). Briefly, we incubated mouse heart sections with mouse IgG-blocking solution from the Mouse on Mouse (M.O.M.TM) kit (Vector Lab) diluted in 0.01% Triton X-100/PBS at room temperature for 1hr, and then incubated the heart sections with 5% goat serum (Sigma-Aldrich) in M.O.M. protein diluent for 30 min. After that, we incubated heart sections with mouse anti-Tsg101 (1:100, Thermo) and rabbit anti-Dystrophin (1:100, Thermo) antibodies diluted in M.O.M. protein diluent at 4°C overnight. In next day, we wash slides with PBS and incubated heart sections with goat anti-mouse Alexa Fluor 555-conjugated and goat anti-rabbit Alexa Fluor 488-conjugated (1:400, Life Technologies, Carlsbad, CA) diluted in M.O.M. protein diluent at room temperature for 45 minutes. After washing and mounting sections with VectaShield Mounting Medium with DAPI, we took pictures with a Zeiss 780 Upright confocal microscope.

Statistical Analysis

All values are expressed as mean $\pm$ standard error of mean (SEM). Student's t-test was used to compare two groups. A value of $p < 0.05$ was considered to indicate statistically significant differences.

Results:

Characterization of MPC

To characterize the MPC (C2C12 cells), we examined MyoD, a skeletal muscle-specific transcription factor, by immunofluorescence confocal microscopy. We detected expression of MyoD in nearly all C2C12 cells (**Fig. 1A**). We also detected robust expression of dystrophin in the cell membranes (**Fig. 1B**).

Characterization of MPC derived exosomes

Morphological analysis of the WT-MPC derived pellets using transmission electron microscopy confirmed the presence of exosomes (**Fig. 2A**). The size of isolated exosomes/microvesicles was measured using a ZetaView®, a nanoparticle tracking analyzer for hydrodynamic particle size. As seen in **Fig. 2B**, the modal size of purified particles is around

100nm. Using optimal PBS buffer dilution of 0.05 (diluted 20 × from 1 × PBS), yielding a conductivity of ~ 816 uS/cm, we determined the zeta potentials for MPC exosomes to be -20.81 ± 1.64 mV at 23 °C (**Fig. 2C**).

MPC-Exo carry dystrophin mRNA

To determine whether MPC-Exo carry dystrophin mRNA, we performed RT-PCR. Consistently, we detected evidence of dystrophin mRNA in MPC-Exo (**Fig. 3A**). To quantify cardiac dystrophin gene expression in MDX mouse hearts, we performed qRT-PCR, and observed that intramyocardial administration of the MPC-Exo increased dystrophin mRNA expression about 8 fold in comparison with PBS control (**Fig. 3B**).

Transplantation of MPC-Exo partially restores dystrophin protein expression in MDX mice

Two days after intramyocardial injection of WT-MPC-Exo, confocal immunofluorescent staining demonstrated partial restoration of dystrophin expression with membrane localization (**Fig. 4A-B**), suggesting that local MPC-Exo delivery can transiently restore dystrophin protein in cardiomyocytes of MDX mice. To identify the relationship between exosomes with dystrophin expression, we co-stain dystrophin with exosome marker Tsg101 in hearts of MDX mice, however, we did not observe obvious difference of Tsg101 expression in cardiomyocytes with restored dystrophin expression in MPC-Exo treated hearts in comparison with cardiomyocytes without dystrophin expression in PBS treated hearts (**Fig. 4C-D**).

Transplantation of MPC-Exo improves left ventricular function in MDX mice

To determine whether transplantation of MPC-Exo can improve myocardial function in MDX mice, we measured contractile function with echocardiography at 48 hrs post-intramyocardial MPC-Exo or PBS delivery. Compared with PBS, MPC-Exo administration improved both left ventricular ejection fraction (EF $74.3 \pm 2.5\%$ vs $58.2 \pm 3.4\%$, $P < 0.05$, $n=6$) and fractional shortening (FS $42.5 \pm 2.4\%$ vs $30.0 \pm 2.3\%$, $P < 0.05$, $n=6$) in MDX mice (**Fig. 5**). Taken together, these observations suggest that MPC-Exo can restore dystrophin expression and may have therapeutic effects to improve heart function in MDX mice.

Discussion:

Our results suggest that MPC-Exo carry dystrophin mRNA, and that MPC-Exo transplantation can increase expression of the full-length dystrophin gene in recipient hearts of MDX mice. Partial restoration of cardiac dystrophin gene expression in MDX mice was associated with transient improvement in heart function.

We observed that MPC-Exo transplantation transiently increased the full-length expression of dystrophin mRNA and restored dystrophin protein expression, appropriately localized to the cell membrane by immunofluorescence staining in the hearts of MDX mice. Thus, local administration of allogeneic MPC-Exo appears to temporarily restore dystrophin gene expression in some cardiomyocytes. This finding is in concordance with the recent report from Aminzadeh MA et al(7), which showed partial restoration of dystrophin after intramyocardial injection of cardiosphere-derived cell (CDC)-derived exosomes in the heart

1 and 3 weeks after systemic injection. The authors suggested that exosome-mediated transfer of miR-148a could be a mechanism of enhanced dystrophin expression (7). Since we found that MPC also carry dystrophin mRNA, we postulate that transfer of dystrophin mRNA potentially could contribute to restoring dystrophin expression in MDX hearts. Moreover, we cannot exclude protein transfer as another mechanism for partial restoration of dystrophin expression in MDX hearts.

Several strategies have been developed to attenuate the symptoms of DMD. Dietary taurine can increase taurine content in muscle of MDX mice, thereby reducing inflammation and improving muscle function(17). Messina S et al(18) reported that flavocoxid counteracts muscle necrosis and improves functional properties in MDX mice via its anti-oxidant and anti-inflammatory properties. Ataluren is a small molecule compound that interacts with ribosomes to block premature nonsense stop signals, thereby enabling production of the full-length, functional protein. In a multicenter, randomized, double-blind, placebo-controlled, phase 3 trial, Ataluren improved function in DMD patients with baseline 6 minute walk distance of 300m~400m compared with placebo(19). Gene therapy has also been developed to attempt to restore dystrophin production in muscle. Since the full-length dystrophin gene is too large to package efficiently into viral vectors, Satamoto M et al. (20) tested the effects of micro-dystrophin cDNA transgene deliver. Delivery of micro-dystrophin gene was observed to improve the muscle phenotype in MDX mice. rAAV-mediated delivery of the follistatin gene, a potent myostatin antagonist, showed preliminary efficacy in reducing endomysial fibrosis and central nucleation, normalizing fiber size distribution, and promoting muscle hypertrophy in a phase 1/2a clinical gene therapy trial in patients with Becker muscular dystrophy(21). Eteplirsen (Exondys 51), the first globally approved drug for DMD, is an antisense oligonucleotide to induce exon 51 skipping. It was effective at increasing dystrophin levels in muscle tissues of patients with DMD(22). Finally, CRISPR gene editing is a novel, promising strategy to correct the DNA mutation in patients with DMD. Zhang Y. et al.(23) recently demonstrated that CRISPR-Cpf1 mediated gene editing of human iPSC can correct the non-sense mutation and restore dystrophin gene expression after differentiation of iPSCs into cardiomyocytes. The CRISPR gene correction allows complete exon correction to fully restore full-length dystrophin expression(24).

Transplantation with allogeneic MPC-derived Exo has its limitations. Exosomes may have major histocompatibility complex class I & II (MHC I& II) antigens in their membranes(25), which could elicit an immune response, and thus the development of autologous MPC exosome strategies may have offer certain advantages for long-term improvement of cardiac function. Recent studies have shown that the CRISPR-Cas9 system can be used to correct dystrophin gene mutations(24). Therefore, genetically correcting dystrophin gene mutations in autologous MPCs using CRISPR gene editing might be a more effective strategy to obtain Exo that could be repeatedly administered to produce long-term beneficial effects at an acceptable risk(26).

Although MPC-Exo is a naturally occurring dystrophin mRNA carrier for DMD treatment, the therapeutic efficiency is determined by the dosage, uptake, and retention of exosomes in hearts. DMD affects whole heart, therefore, selection of delivery approach is critical for success of exosome therapy in treating DMD. There are three common routes for exosome

delivery: local injection, intracoronary infusion, and intravenous infusion. The local injection has the advantage of delivering large amount of exosomes to hearts with good retention, recent a meta-analysis reported the superiority of transendocardial stem cell injection in reduction in infarct size and improvement of left ventricular ejection fraction (LVEF)(27). We also found that this approach can improve heart function in hearts of MDX mice. However, DMD is a chronic disease with inflammatory cell infiltration, repeated exosome delivery is necessary to inhibit inflammation and fibrosis, and preserve heart function. Both local injection and intracoronary injection are invasive approaches, which are hard to be accepted by most of patients for repeated treatment due to the transient effect of exosomes. Systemic injection, such as intravenous injection (i.v.), is widely accepted by patients for repeated drug delivery, however, most of i.v. delivered exosomes will be distributed in whole body, and might accumulate preferentially in liver, lung, kidney and spleen, and then rapidly cleared from body in these tissues. To avoid off-target effects with using systemic route, we need modify exosomes to improve their cardiac homing capacity. Exosome targeting can be achieved by modifying surface of exosomes with cardiac targeting peptide, such as CRPPR, which is a cardiac homing peptide/receptor pairs identified by ex vivo/in vivo phage display and bacterial 2-hybrid analysis(28). In addition, the exosome homing can be improved by modification of targeting peptides to protect them from protease degradation. Kim H et al(29) recently used cardiac-targeting peptide (CTP) Lamp2b to modify exosome, and found that this modification can increase in vivo exosome delivery to heart tissue by 15%, suggesting that exosomes can be modified to suit for systemic route of delivery.

Limitations:

Due to the technique limitation, we cannot track the whole process of packed mRNA in exosomes converting to dystrophin protein in cardiomyocytes. To directly track the process, we can use CRISPR/Cas9 knock-in technology to knock in protein Tags, such as Flag peptide, into exon of dystrophin gene to allow exosome derived exogenous dystrophin be tagged. After MPC-Exo transplantation, we can use anti-Flag antibody to detect exogenous dystrophin protein in recipient cardiomyocytes, if we observed Flagged dystrophin protein in recipient cardiomyocytes of MDX mice, it will be direct evidence that mRNA packed in exosomes can be converted to dystrophin protein in recipients.

Moreover, we cannot exclude the possibility that other mRNAs packed in the exosomes contributing to cardiac function improvement. To identify other critical mRNA in MPC-Exo, we should first do RNA-seq to identify the most abundant RNAs in MPC-Exo, and then use siRNA to knock-down individual high abundant mRNA to determine their role in improving cardiac function.

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Statement of clinical relevance:

Progressive cardiomyocyte loss in Duchenne muscular dystrophy (DMD) leads to cardiac fibrosis, in this study, we observed that allogeneic WT-MPC-Exo transplantation transiently restored dystrophin gene expression and improved cardiac function in MDX mice, therefore, allogenic exosomal delivery may serve as an alternative treatment for cardiomyopathy of DMD. In our opinion, the exosome therapy might be used for releasing syndrome for short term, and repeated treatment is necessary. The CRISPR gene editing is a powerful, promising technology for final cure, however, the efficiency of CRISPR-Cpf1 is still quite low, and CRISPR technology has concern of high off-target effect. Therefore, exosome therapy using exosomes from myogenic progenitor cells might be an option for DMD patients.

Fig. 1. Immunophenotype characterization of C2C12 cells grown in vitro. Cells were counterstained with DAPI (blue). **(A)** C2C12 cells expressed MyoD, a myogenic transcription factor and marker of myogenic progenitors, in their nuclei. **(B)** Immunofluorescent imaging demonstrated that most C2C12 cells express Dystrophin in their membranes.

Fig. 2. Characterization of MPC-exosomes/microvesicles. **(A)** Electron micrograph image of C2C12-derived exosomes/microvesicles. The image shows round, cup-shaped vesicles. Scale bar = 200 nm. **(B)** Particle size distribution in purified pellets consistent with size range of exosomes/microvesicles (average size around 100 nm), measured by ZetaView® Particle Tracking Analyzer. **(C)** Zeta potential of MPC-Exo at 25 °C.

Fig. 3.

Dystrophin mRNA in MPC-Exo and recipient hearts of MDx mice. **(A)** Detection of dystrophin mRNA in WT-MPC-Exo; **(B)** Dystrophin transcripts in hearts of MDX mice treated with either PBS or MPC-Exo. qRT-PCR results show increased dystrophin mRNA transcription (normalized to GAPDH levels) in MPC-Exo treated hearts of MDX mice in comparison to PBS treated control hearts (*, $P < 0.05$, $n = 3$).

Fig. 4. Cross sectional images of the hearts of MDX mice 2 days after PBS or MPC-Exo treatment. (A) MPC-Exo treatment led to partial restoration of dystrophin protein expression in the heart; (B) 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, *, $P < 0.05$); (C) co-staining of Tsg101 with Dystrophin in PBS treated hearts of MDX mice; (D) co-staining of Tsg101 with Dystrophin in MPC-Exo treated hearts of MDX mice.

Fig. 5. Echocardiographic measurements of cardiac function 2 days after PBS or MPC-Exo treatment. Left-ventricular ejection fraction (EF) and fractional shortening (FS) were significantly higher in mice treated with MPC-Exo in comparison with PBS (*, $P < 0.05$, $n = 6$).

Table 1:

Prime list

Gene list	Sequence (5'-3')
Dystrophin FWD	ATG AAG GTG GGC AGA AGA TAA A
Dystrophin REV	TGT GAT CCC ACT GAG TGT TAA G
GAPDH FWD	AGTGGGAGTTGCTGTTGAAG
GAPDH REV	TGACATCAAGAAGGTGGTGAAG