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Exosomes-Based Gene Therapy for MicroRNA Delivery

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Abstract

Despite recent advances in scientific knowledge and clinical practice, cardiovascular disease management and treatment remain a major burden. While several treatment strategies using drugs and surgeries are being developed for cardiovascular manifestations, gene-based therapies hold significant promise. Recent findings from our laboratory unveiled a novel mechanism that exosomes, secreted nanovesicles from stem cells, mediate cardiac repair via transferring their unique repertoire of microRNAs (miRNA) to recipient cells in the heart. Exosomes, unlike other vectors for gene delivery, present unique advantages such that exosomes are a cell-free natural system for ferrying RNA between cells, robust exosomal membrane can protect the RNA/gene of interest from digestion, and exosomes are rapidly taken up by target cells making them a more efficient vehicle for gene delivery. Here, we describe a stepwise protocol developed in our laboratory for generating exosomes from human CD34⁺ stem cells that carry exogenously applied Cy3 dye-labeled pre-miR miRNA precursors. We demonstrate that human CD34⁺ stem cell exosomes can rigorously enter into recipient cells and deliver Cy3 dye-labeled pre-miR miRNA precursors to regulate gene expression. Identification of key molecular targets to treat disease conditions is the foremost critical step and the novel approach presented here to generate exosomes carrying exogenous genetic information offers a valuable clinical tool for more effective treatment strategies.

Keywords

Gene therapy; CD34⁺ stem cells; Exosomes; Microvesicles; MicroRNA

1 Introduction

Almost after three decades since first report, exosomes are now recognized as vital mediators of cell–cell communication. Exosomes are extracellular vesicles of endocytic origin secreted by almost every cell type in humans and typically range in 30–100 nm size [1]. Exosomes carry macromolecules including lipids, proteins, and nucleic acids (mainly RNA) and their composition reflects their parent cell [2]. In the past few years, the exosomes field has grown exponentially both in the aspect of fundamental cell biology as well as pathologies involving cancer and cardiovascular diseases [3]. Indeed, secreted exosomes containing biologically active macromolecules can selectively bind to cell surface receptors presented by recipient cells. Upon receptor binding, exosomes are thought to transduce specific intracellular signaling thereby inducing physiological changes in recipient cells [4]. Another mechanism by which exosomes mediate biological information is through direct

transfer of intra-exosomal content such as mRNA and microRNA (miRNA or miR) inside recipient cells by fusion with cell membrane [5]. Herein, in-depth understanding of exosomes-mediated biological information transfer is a critical step towards gaining deeper insights to exosomes function in both physiology and pathophysiology.

MiRNAs are critical regulators of gene expression at the post-transcriptional level in higher eukaryotes [6]. Regulation of miRNA expression is tightly coordinated in a developmental and tissue-specific fashion [7]. Perturbations to miRNA expression lead to severe developmental anomalies [8]. Several miRNAs have been implicated to have role in chronic diseases and conditions such as cancer, heart and neurodegenerative diseases [9]. Consequently, miRNAs are regarded as potential pharmacological candidates for therapeutic treatment as well as for biomarker discoveries in heart failure [10]. For instance, expression of miRNA is deregulated under pathological heart conditions and a major pharmacological interest is to restore endogenous miRNA expression either through the use of miRNA “mimics” or “inhibitors” under disease states [11, 12]. However, a major limitation is the lack of efficient cell-specific gene delivery system as well as off-target toxicity effects. Adeno-associated virus (AAV) is one of the efficient delivery methods for gene therapy that has been widely in practice for long time. In the treatment of heart failure, AAV-mediated gene delivery for genes implicated in cardiac contractility has significant clinical value [13]. A major limitation in using viral vectors is the existence of endogenous neutralizing antibodies in human sera. In addition, viral-mediated vectors are prone to entering of undesired cell and tissue types when introduced intravenously or intramuscularly. To successfully achieve high efficient delivery of key genes for therapeutics, choice of reliable gene delivery method is the foremost critical step. Our approach to overcome such limitations is through modulation of exosomes by targeted approach as a reliable gene delivery system for the expression and function of miRNAs in recipient cells. Indeed, exosomes offer unique advantage over several gene delivery systems for at least two reasons. One of that is exosomes are natural carriers of biological information in the form of miRNA and can deliver miRNA content to specific cell types via receptor-mediated binding. Secondly, exosomes are rapidly taken up by recipient cells, therefore minimal off-target effects are exhibited through systemic circulation.

Recent discoveries highlight that exosomes selectively carry miRNAs in abundance and mediate crosstalk between different cell types by direct transfer of miRNA in to recipient cells [14]. For example, human CD34⁺ stem cell-derived exosomes carry miRNAs that are implicated in proangiogenesis and ischemic tissue repair [15]. This natural exosomes-mediated communication can be harnessed to deliver exogenous genetic material to further enhance the success and outcomes of gene therapy [16]. In this Chapter, we will demonstrate how exosomes can be modulated using exogenous Cy3 dye-labeled pre-miR miRNA precursors, which are miRNA mimics that will enable direct transfer of pre-miR miRNA precursors into recipient cells via exosomes uptake (Fig. 1). Throughout this protocol, we use Cy3 dye-labeled pre-miR miRNA precursors to monitor exosomes uptake in real time using fluorescent imaging technique. We further demonstrate using flow cytometry analysis that human CD34⁺ stem cell-secreted exosomes predominantly carry Cy3 dye-labeled pre-miR miRNA precursors. We then show using fluorescent imaging that human umbilical vein

endothelial cells (HUVEC) specifically uptake CD34⁺ stem cell exosomes containing Cy3 dye-labeled pre-miR miRNA precursors.

2 Materials

All aqueous solutions, buffers, reagents, H₂O, tips, and pipettes used in this protocol are molecular biology grade, sterile, and RNase/DNase-free certified.

2.1 General Laboratory Materials

- 15 and 50 ml polypropylene conical tubes.
- 10 and 25 ml serological pipettes.
- RNase/DNase-free pipettes and tips.
- Sterile non-latex gloves.
- Sterile RNase/DNase-free 1.5 ml eppendorf tubes.
- RNaseZap Wipes.
- 70% ethanol.
- Alcohol wipes.

2.2 Cell Culture

- Human CD34⁺ stem cells isolated from peripheral blood of GCSF-mobilized individuals.
- GFP-expressing human umbilical vein endothelial cells (HUVEC).
- X-VIVO 10 medium.
- Human serum albumin.
- Human recombinant FLT-3 ligand protein (FLT-3L).
- Human recombinant stem cell factor protein (SCF).
- Human recombinant vascular endothelial growth factor (VEGF).
- Phosphate buffered saline (1×) without Ca²⁺ and Mg²⁺.
- T25 and T75 cell culture flasks (cell culture treated).
- 24-well culture plates.
- Trypan blue.
- Hemocytometer.
- EBM2 media with MV SingleQuots.

2.3 Cy3 miRNA Transfection, Exosomes Isolation, and HUVEC Uptake

- Cy3 dye-labeled pre-miR miRNA precursors.

- Opti-MEM Medium.
- Lipofectamine RNAiMAX Reagent.
- RNase A enzyme.
- RNase inhibitor.
- Polycarbonate ultracentrifuge tubes with aluminum cap assembly for the Type 70 Ti rotor.
- Bottlebrush.
- Soap.
- 4 μm latex Beads.
- FACS buffer (1 $\times$ sterile PBS, 0.5–1 % BSA or 5–10 % FBS, 2 mM EDTA).
- Protease- and nuclease-free sucrose.
- Tris base.
- D₂O.
- 10 N HCL.
- Sterile 0.22 μm filter.
- 16-well chambered cover glass for cell culture.

2.4 Equipment

Cell culture incubator capable of 37 °C, 5 % CO₂ and 85 % relative humidity.

pH meter.

Inverted cell culture microscope.

Confocal microscope.

Small bench top low-speed centrifuge.

High-speed centrifuge.

Ultracentrifuge with Type 70 rotor.

Laminar flow hood for sterile techniques.

Flow cytometer.

Dynamic light scattering equipment for nanoparticle size characterization.

3 Methods

Always work under sterile conditions inside the laminar flow hood. All of the described steps in this protocol including Human CD34⁺ stem cell culture, Lipofectamine-mediated transfection of Cy3 dye-labeled pre-miR miRNA precursors, exosomes isolation and exosomes uptake by HUVECs require sterile cell culture practice. Use of sterile, RNase/

DNase-free eppendorf tubes and polypropylene conical tubes are highly recommended as miRNA is subject to degradation by ubiquitously present RNase enzyme and will interfere with transfection efficiency.

Work in a laminar flow hood designated as RNase-free zone and always wipe with ethanol and RNaseZap wipes at regular intervals in between performing steps.

3.1 Human CD34⁺ Stem Cell Culture

1. Warm X-VIVO 10 medium in a 37 °C tissue culture water bath.
2. Dissolve each tube of FLT-3L, SCF and VEGF in XVIVO (1 ml or as required) for preparing complete X-VIVO 10 medium on ice.
3. Prepare X-VIVO 10 medium (complete) for CD34⁺ stem cell culture by adding

Human Serum Albumin	0.25%
FLT-3L	100 ng/ml
SCF	100 ng/ml
VEGF	20 ng/ml

4. It is strongly recommended that fresh complete media is prepared on the day when the experiment is run.
5. Prepare cells for counting and establish an estimated number of total CD34⁺ stem cells using trypan blue cell staining in a hemocytometer under microscope.
6. In a T75 flask, plate human CD34⁺ stem cells at a cell density of 250,000–300,000 cells/ml using complete X-VIVO 10 medium. A minimum of 2×10^6 cells is required for exosomes preparation.
7. Culture cells in a cell culture incubator capable of 37 °C, 5 % CO₂ and 85 % relative humidity for 24–48 h to achieve 70–80 % confluence.

3.2 Lipofectamine- Mediated Transfection of Cy3 Dye-Labeled pre-miR miRNA Precursors in Human CD34⁺ Stem Cells

Cy3 dye-labeled pre-miR miRNA precursors must be protected from light. Ensure that the tubes and cell culture flasks containing Cy3 dye-labeled pre-miR miRNA precursors are protected from light. Excessive exposure to light can interfere with the quality of fluorescent imaging analysis.

1. On the day of transfection, collect media containing human CD34⁺ stem cells. Ensure maximal cell recovery by gently flushing new complete X-VIVO media into T75 flask and collect remaining cells.
2. Centrifuge the media containing CD34⁺ stem cells in 15 ml polypropylene conical tubes at $400 \times g$ for 7 min. Discard the supernatant. This will remove the cell debris and exosomes secreted during this period of cell culture.

3. To the cell pellet, add freshly prepared 1–2 ml X-VIVO 10 (complete) media and mix gently.
4. In a sterile 24-well culture plate, dilute Lipofectamine by mixing gently 150 μ l Opti-MEM medium with 9 μ l Lipofectamine in one well. Prepare this mix in at least 8 wells of a 24-well culture plate.
5. In a new well, dilute Cy3-labeled pre-miR miRNA precursors in Opti-MEM medium by mixing gently 3 μ l Cy3 pre-miR (10 μ M stock) with 150 μ l Opti-MEM medium. Prepare this mix separately in at least 8 wells.
6. Transfer diluted Lipofectamine (~159 μ l) to each well containing Cy3 pre-miR (~153 μ l) and mix gently.
7. Incubate the miRNA-lipid complex in room temperature for 5 min.
8. To each well containing Lipofectamine and Cy3 pre-miR mix, add 1 ml of X-VIVO medium containing 250,000–300,000 human CD34⁺ stem cells. Swirl the 24-well plate gently to mix contents.
9. Allow reverse transfection to occur by leaving cells in incubator for at least 24 h at 37 °C.
10. After 24 h of transfection, collect cells in a 15 ml sterile polypropylene conical tube.
11. Centrifuge 50 ml polypropylene conical tubes containing CD34⁺ stem cells at 400 $\times g$ for 7 min. Discard the supernatant. This will remove the free Cy3 dye-labeled pre-miR miRNA precursors, non-transfected lipid-miRNA complexes, cell debris and exosomes secreted during this period of cell culture.
12. To the cell pellet, add freshly prepared X-VIVO 10 (complete) media and mix gently.
13. Plate these cells in a fresh T25 flask at a cell density of 250,000–300,000 cells/ml using complete X-VIVO 10 medium.
14. Leave the transfected CD34⁺ stem cells in culture for up to 30–36 h in an incubator at 37 °C, 5 % CO₂ and 85 % relative humidity for Cy3 miRNA secretion via exosomes.
15. At this stage, Cy3-transfected cells can be visualized using fluorescent microscopy (Fig. 2). The transfection efficiency is generally 60–80 %.

3.3 Isolation and Purification of Cy3 miRNA-Containing Exosomes from CD34⁺ Stem Cells

1. The day before exosomes isolation, wash the bottom of polycarbonate ultracentrifuge tubes inside with hot water and soap using a bottlebrush.
2. Place washed glass ultracentrifuge tubes inside laminar flow chamber to maintain sterile condition inside the tubes.

3. Spray 5 ml of 70 % ethanol alongside the inner walls of ultracentrifuge tubes and close the lid. Shake and invert several times to wash all sides of the tube as well as the lid.
4. Aspirate out ethanol and leave the tubes open overnight inside laminar flow chamber to let residual ethanol evaporate.
5. On the day of exosomes isolation, wash polycarbonate ultracentrifuge tubes using 5 ml of sterile PBS.
6. Turn on the ultracentrifuge (with the Ti70 rotor inside) and chill to 4 °C with the “vacuum” mode. This will take approximately 2–3 h.
7. Set ultracentrifuge to $100,000 \times g$, time 60–75 min.
8. Following 30–36 h of Cy3-transfected human CD34⁺ stem cell culture, collect transfected cells and conditioned media together using serological pipette in a fresh 15 ml polypropylene conical tube.
9. In a small bench top low-speed centrifuge, place 15 ml polypropylene conical tube containing transfected CD34⁺ stem cells and conditioned media and centrifuge at $500 \times g$ for 10 min at 4 °C to separate cells from the conditioned media.
10. Carefully transfer the supernatant containing conditioned media to a fresh 15 ml polypropylene conical tube and leave on ice. Freeze the cell pellet at –80 °C or culture in fresh X-VIVO 10 (complete) media if required for further analysis.
11. Centrifuge the supernatant containing exosomes at $2000 \times g$ for 15 min at 4 °C to remove cell fragments and debris [17].
12. In a high-speed centrifuge, place the conical tubes containing conditioned media and centrifuge at $14,000 \times g$ for 20 min. This will pellet large cell debris, apoptotic bodies and other undesired extracellular vesicles leaving the soluble exosomes in the supernatant. Carefully transfer the supernatant to a fresh conical tube.
13. Prepare Tris/sucrose/D₂O solution as follows
 - 30 g protease- and nuclease-free sucrose.
 - 2.4 g Tris base.
 - 50 ml D₂O.
 - Adjust pH to 7.4 with 10 N HCL drops.
 - Adjust volume to 100 ml with D₂O.
 - Sterilize by passing through 0.22 µm filter.
 - Store up to 2 months at 4 °C.
14. Load 4 ml of ice-cold Tris/sucrose/D₂O solution at the bottom of an ultracentrifuge tube. This will make a cushion/floatation of exosomes.

15. Layer the supernatant containing exosomes from step 12 (of this section) on top of the sucrose cushion. Layering must be done gently without disturbing the sucrose cushion interface. Bring the volume up to the neck of the tube. Balance tubes by adding ice-cold PBS (w/o Ca^{2+} and Mg^{2+}). Tighten the lids (*see* Note 1).
16. Ultracentrifuge the tubes at $100,000 \times g$ for 60–75 min at 4 °C.
17. Remove supernatant and collect ~4 ml sucrose cushion, which now contains exosomes. Only collect the sucrose cushion and do not contaminate with pellet, if there is any.
18. Transfer the sucrose (~4 ml) to a fresh sterile polycarbonate ultracentrifuge tube. Bring the volume up to the neck of the tube using ice cold PBS (w/o Ca^{2+} and Mg^{2+}). Balance tubes by adding ice-cold PBS. Mix well. The mass of the tubes should be within 0.03 g. Ensure the tubes are precisely balanced using a bench top weighing balance.
19. Ultracentrifuge the tubes at $100,000 \times g$ for 60–75 min at 4 °C.
20. The exosomes pellet is extremely fragile and easily disturbed by minimal physical distractions and can lead to significant loss in exosomes quantity. Therefore utmost care must be taken when aspirating the supernatant leaving the exosomes pellet intact.
21. Aspirate supernatant and resuspend pellet in 50–100 μl ice-cold PBS and carefully transfer to fresh sterile RNase/DNase-free eppendorf tubes.
22. Exosomes can be frozen for future experiments or can be used immediately for downstream analysis.

3.4 Exosomes Quality Analysis and Preparation for Gene Delivery

1. To avoid precipitation of undesired RNA molecules sticking on to exosomal outer membranes, it is recommended to perform RNase A digestion of isolated exosomes sample.
2. Add RNase A enzyme to exosomal preparation at a 100 $\mu\text{g}/\text{ml}$ final concentration and mix gently.
3. Incubate at 37 °C for 15 min.
4. To avoid RNase A carryover throughout further steps, addition of RNase inhibitor is recommended.
5. Add RNase Inhibitor to the mixture 1 U/ml and incubate at 37 °C for 10 min.
6. In a fresh ultracentrifuge tube, load 4 ml of ice-cold Tris/ sucrose/ D_2O solution at the bottom of an ultracentrifuge tube.

¹Layering of exosomes sample on top of sucrose cushion needs caution when performing. A minimal disruption to exosomes- sucrose interface can significantly affect exosomes quality and quantity after ultracentrifugation. Such disruptions can introduce unwanted protein and large lipid complexes as contaminants in exosomes preparation thereby affecting exosomes purity.

7. Carefully layer the RNase A treated exosomes sample on top of the sucrose cushion. Bring the volume up to the neck of the tube. Balance tubes by adding ice-cold PBS (w/o Ca^{2+} and Mg^{2+}). Tighten the lids.
8. Ultracentrifuge the tubes at $100,000 \times g$ for 60–75 min at 4 °C.
9. Remove supernatant and collect ~4 ml sucrose cushion, which now contains exosomes. Only collect the sucrose cushion and do not contaminate with pellet, if there is any.
10. Transfer the sucrose cushion (~4 ml) to a fresh sterile ultracentrifuge tube. Bring the volume up to the neck of the tube. Balance tubes by adding ice-cold PBS (w/o Ca^{2+} and Mg^{2+}). Mix well.
11. Ultracentrifuge the tubes at $100,000 \times g$ for 60–75 min at 4 °C.
12. Aspirate supernatant and resuspend pellet in 50–100 μl ice-cold PBS and carefully transfer to fresh sterile RNase/DNase-free eppendorf tubes. To determine the intactness of exosomes post-RNase treatment, dynamic light scattering (DLS) analysis can be performed (*see* Notes 2 and 3).
13. In a fresh sterile RNase/DNase-free eppendorf tube, aliquot required volume of 4 μm latex beads (4×10^7 beads/ml). It is recommended to use 200,000 beads per 1×10^6 cells.
14. Wash beads at least two times using FACS buffer (500 μl) and pellet beads each time by centrifugation at $8000 \times g$ for 4 min.
15. Resuspend beads in 100 μl FACS buffer and transfer to a fresh eppendorf tube.
16. To the washed beads, add exosomes prepared from 2×10^6 CD34⁺ stem cells and mix gently.
17. Incubate at room temperature for at least 30 min. Without rocking.
18. Transfer beads + exosomes mixture to a laboratory rocker and incubate for additional 2 h with rocking (*see* Note 4).
19. Centrifuge bead-tagged exosomes at $8000 \times g$ for 4 min.
20. Wash the bead-tagged exosomes pellet by adding 500 μl FACS buffer and centrifugation at $8000 \times g$ for 4 min.
21. Resuspend the pellet (beads + exosomes) in 200 μl FACS buffer.
22. Keep the beads + exosomes mixture on ice.
23. Perform flow cytometry (*see* Note 5). Note that we recommend always including controls such as (1) Beads only, (2) beads + unstained exosomes, and (3) beads +

²If the exosomes sample is limited, exosomes sample used for dynamic light scattering analysis can be reused for downstream procedures such as RNA/protein/flow cytometry analysis.

³During DLS analysis, if there is a significant proportion of particle size greater than 500 nm present, performing an additional sucrose-based gradient ultracentrifugation step is recommended to further deplete contaminating larger particles.

⁴When incubating 4 μm beads with exosomes for uptake experiments with HUVECs, rocking should be performed with high speed at room temperature. This is typically 50–60 RPM.

Cy3 miR-containing exosomes. All these are processed simultaneously and treated identically.

24. FACS analysis will ensure the quality of exosomes purification as well as the enrichment of Cy3-tagged exosomes for downstream gene delivery protocols.
25. As shown in Fig. 3, we confirm efficient transfection of Cy3-labeled pre-miR miRNA precursors to human CD34⁺ stem cells and release of Cy3 miRNA via exosomes secretion. This ensures that CD34⁺ stem cell-derived exosomes can be used for targeted gene delivery.

3.5 Direct Transfer of Cy3 Dye-Labeled pre-miR miRNA Precursors by Human CD34⁺ Stem Cell-Derived Exosomes

1. To monitor Cy3-tagged exosomes uptake by fluorescent microscopy, we used HUVEC cell line that constantly express Green Fluorescent Protein (GFP).
2. Care should be taken to minimize exposure of GFP-tagged HUVEC cells to light throughout the procedure.
3. Culture GFP-expressing HUVECs in a T75 flask using EBM2 media until it reaches 70–80 % confluence.
4. Prepare a sterile 16-well chambered cover glass for exosomes miRNA delivery.
5. Upon 70–80 % confluence, trypsinize GFP-expressing HUVEC cells and perform cell counting by trypan blue staining.
6. Carefully transfer $2-3 \times 10^4$ GFP-expressing HUVEC cells to 16-well chambered cover glass.
7. Normalize exosomes volume to initial CD34⁺ stem cell number, i.e., it is recommended to use exosomes from 1×10^6 CD34⁺ stem cells per $2-3 \times 10^4$ GFP-expressing HUVEC cells. For example, if exosomes were prepared from 3×10^6 CD34⁺ stem cells, use one-third of exosomes sample to transfect $2-3 \times 10^4$ GFP-expressing HUVEC cells in a 16-well chambered cover glass.
8. Cover the 16-well chambered cover glass.
9. Place the 16-well chambered cover glass in a dark cell culture incubator capable of 37 °C, 5 % CO₂ and 85 % relative humidity.
10. Culture HUVECs in presence of exosomes for 12–24 h.
11. Exosomal uptake by HUVECs can be visualized live under a fluorescent/confocal microscope (Fig. 4).

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⁵When performing flow cytometry, it is also recommended to include a positive control by incubating 4 µm beads with Cy3 miRNA only. This control will assist in flow cytometry gating.

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Fig. 1.
Workflow depicting critical stages involved in exosomes-mediated miRNA gene delivery

Fig. 2. Fluorescent microscopy imaging of Lipofectamine-mediated transfection of Cy3 dye-labeled pre-miR miRNA precursors in human CD34⁺ stem cells. Live confocal microscopy imaging at 20× magnification showing efficient transfection of Cy3-labeled pre-miR miRNA precursors in human CD34⁺ stem cells. Cells that appear orange indicate Cy3 dye-transfected human CD34⁺ stem cells (*white arrows*)

Fig. 3.

Fluorescent activated cell sorting analysis of targeted human CD34⁺ stem cell exosomes containing Cy3 dye-labeled pre-miR miRNA precursors. CD34⁺ stem cell derived exosomes were prepared by sucrose gradient centrifugation and coated on to 4 μ m latex beads followed by visualization by flow cytometry. (a) Beads-beads control (b) Beads-beads control exosomes (non-Cy3) (c) Beads-beads Cy3 exosomes

Fig. 4. In vitro monitoring of human CD34⁺ stem cell derived exosomes-mediated delivery of Cy3 dye-labeled pre-miR miRNA precursors in GFP-tagged HUVECs. Visualization of human CD34⁺ stem cell-derived exosomes inside HUVECs. *Red* color is representative of Cy3 miRNA containing exosomes after entering HUVEC cells. HUVEC cells expressing green fluorescent protein