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Mining Exosomal microRNAs from Human Induced Pluripotent Stem Cells-Derived Cardiomyocytes for Cardiac Regeneration

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INTRODUCTION

Cardiovascular diseases (CVD) are the primary cause of morbidity and mortality worldwide [1]. Following myocardial infarction, there is a major loss of cardiomyocytes due to necrosis, and replacement of damaged myocardium is an intense area of research in the field of cardiac regenerative medicine [2]. Various cell types, including mesenchymal stem cells (MSCs), cardiac progenitor cells (CPCs), embryonic stem cells-derived cardiomyocytes (ESC-CMs), and induced pluripotent stem cells-derived cardiomyocytes (iPSC-CMs), have been investigated in both preclinical and clinical studies with mixed results, with a common issue being the poor survival of transplanted cells [3–7]. Positive outcomes of such studies are been associated with the secretion of paracrine factors in the absence of adequate cell survival, which may be mediated by the release of a novel class of small vesicles termed exosomes [7].

Exosomes are endosomal membrane vesicles with diameters of ~40–150 nm that originate in the late endosomal compartment from the inward budding of endosomal membranes, leading to the formation of intracellular multi-vesicular endosomes (MVEs) [8,9]. Groups of exosomes are packed in the MVEs and upon their fusion with the plasma membrane, these exosomes are released into the extracellular space. Various studies have reported the role of exosomes in mediating intracellular communication by serving as vehicles for transferring various biological molecules, including DNA, RNA, proteins, and lipids between cells [10–13]. Consistent with these studies, the roles of exosomes isolated from various types of stem cells such as MSCs, CPCs, ESCs, iPSCs and CD34⁺ have been investigated in mediating cardiac regeneration. These exosomes are known to possess anti-apoptotic effects in both host as well as transplanted cells, and they also stimulate angiogenesis, reduce infarct size, and improve cardiac recovery [14–18]. Importantly, enrichment of protective microRNAs appears to be responsible for the beneficial effects of exosomes in cardiac regeneration [14,10,19,20].

In the following text, we have outlined a protocol for differentiating human iPSCs into cardiomyocytes, and described the methods required for isolating exosomes secreted by

iPSC-CMs for subsequent microRNA analysis, which will help advance our understanding of exosomes and how they can be used for mediating cardiac regeneration moving forward.

MATERIALS

Cultivation and Passaging of Human iPSCs

1. Essential 8 medium (ThermoFisher)
2. BD Matrigel
3. ROCK Inhibitor, Y-27632
4. DMEM/F12 media
5. 0.5M EDTA
6. Dulbecco's phosphate-buffered saline (DPBS) 1x, without $\text{Ca}^{2+}/\text{Mg}^{2+}$
7. Tissue culture plates, 6-well
8. Humidified 5% CO_2 incubator

Cardiac Differentiation of Human iPSCs

1. RPMI 1640 medium (ThermoFisher)
2. RPMI 1640 medium, no glucose (ThermoFisher)
3. B27 Supplement (50x), serum free (ThermoFisher)
4. B27 Supplement (50x), minus insulin (ThermoFisher)
5. CHIR99021
6. IWR-1
7. Tissue culture plates, 6-well
8. Humidified 5% CO_2 incubator

Hydration and Saturation of Filter Units for Concentration

1. Amicon Ultra-15 centrifugal units, 100 kDa (Millipore)
2. Dulbecco's phosphate-buffered saline (DPBS) 1x, without $\text{Ca}^{2+}/\text{Mg}^{2+}$
3. Bovine serum albumin
4. Centrifuge

Preparation and Concentration of iPSC-CMs Conditioned Medium

1. Conical tubes, 50 ml
2. Refrigerated centrifuge
3. Optional: exosome-depleted FBS
4. 0.22 μM filter units

5. Hydrated 100 kDa centrifugal units

Isolation of Exosomes

1. Total exosome isolation reagent from cell culture media (ThermoFisher)
2. Fridge
3. Refrigerated centrifuge
4. Dulbecco's phosphate-buffered saline (DPBS) 1x, without $\text{Ca}^{2+}/\text{Mg}^{2+}$
5. Eppendorf tubes

RNA Isolation from Exosomes

1. miRCURY™ RNA isolation kit – cell & plant (Exiqon)
2. Centrifuge
3. 96–100% ethanol
4. β -mercaptoethanol (add 10 μl per 1 ml lysis buffer)
5. RNase-free water

METHODS

Cultivation and Passaging of Human iPSCs

1. Thaw an aliquot of Matrigel at 4°C, and dilute it at 200–300 times in DMEM/F12 media. Mix well and add 1 ml of Matrigel solution per well into 6-well plates.
2. Incubate the Matrigel-coated plates in a 37°C cell culture incubator for at least 30 min prior to use, plates can be kept for several days before use.
3. Prepare essential 8 (E8) medium by adding 10 ml of supplement into 500 ml of basal medium, and keep it at 4°C for up to 2 weeks. Allow the medium to reach room temperature prior to use.
4. Thaw a vial of frozen iPSCs, and add the thawed cell suspension into 4 ml of E8 medium in a sterile 15 ml conical tube.
5. Centrifuge tube at 300 g for 3 min at room temperature.
6. Aspirate supernatant and gently resuspend cells into 2 ml of E8 medium supplemented with 10 μM of Rho-associated protein kinase (ROCK) inhibitor (S1049, Selleck Chemicals).
7. Plate cells in 1 well of 6-well plate and incubate the cells in a 37°C 5% CO_2 incubator.
8. Replace with fresh medium without ROCK inhibitor the next day, and continue maintaining the cells with daily medium exchange until ~85% confluency (typically 4 days after plating).

9. Cells can then be passaged at split ratios between 1:6 to 1:12. For passaging, aspirate the media from cells to be passaged and wash briefly with 1 ml of PBS (without Ca^{2+} and Mg^{2+}). Aspirate the PBS wash.
10. Add 1 ml of room temperature 0.5 mM EDTA solution to the cells and incubate at room temperature for 5–6 min. During the wait, prepare 24 ml of E8 medium with 10 μM of ROCK inhibitor.
11. Carefully aspirate the EDTA solution from the well without rinsing the cells.
12. Take 1 ml out of the 24 ml of E8 medium with 10 μM of ROCK inhibitor and add gently into the well to dislodge the cells, resuspend 1 to 2 more times if needed.
13. Recover the 1 ml cell suspension and add it back to the remaining 23 ml of E8 medium with 10 μM of ROCK inhibitor.
14. Resuspend gently once, and pipet 2 ml of cell suspension into each well of two 6-well plates for a 1:12 split ratio.
15. Replace cell culture media daily as mentioned in step 8 and passage as needed.

Cardiac Differentiation of Human iPSCs

1. Cardiac differentiation is initiated when iPSCs are between 70–85% confluency. Aspirate the spent E8 medium, and replace with 2 ml of RPMI/B27-Ins medium supplemented with 8 μM of CHIR99021 (day 0) (S2924, Selleck Chemicals). Return cells to the 37°C 5% CO_2 incubator for 48 hours.
2. On day 2, aspirate the spent medium and replace with 2 ml of RPMI/B27-Ins medium. Return cells to the incubator.
3. On day 3, aspirate the spent medium and replace with 2 ml of RPMI/B27-Ins medium supplemented with 5 μM of IWR-1 (S7086, Selleck Chemicals). Return cells to the incubator for 48 hours.
4. On day 5, aspirate the spent medium and replace with 2 ml of RPMI/B27-Ins medium. Return cells to the incubator for 48 hours.
5. On day 7, aspirate the spent medium and replace with 2 ml of RPMI/B27+Ins medium. Return cells to the incubator for 48 hours.
6. On day 9, aspirate the spent medium and replace with 2 ml of RPMI/B27+Ins medium. Return cells to the incubator for 24 hours.
7. On day 10, aspirate the spent medium, and glucose starvation (for eliminating contaminating cells) is started by the addition of 2 ml of RPMI no glucose/B27+Ins medium. Return cells to the incubator and repeat this step every 2 days.
8. On day 15 (end of glucose starvation), aspirate the spent medium and replace with 2 ml of RPMI/B27+Ins medium. Return cells to the incubator and repeat this step every 2 days. Spontaneously contracting troponin T cardiac type 2

positive cardiomyocytes will be present and ready for use, or can be further cultured as desired.

Hydration and Saturation of Filter Units for Concentration

Amicon columns of 50 kDa or 100 kDa from Millipore or Vivaspin columns from Sartorius are recommended, with polyethersulfone (PES) as membranes.

1. Columns are washed with 10 ml of PBS (1x) at 3000 g for 20 min at room temperature.
2. Membrane is then saturated with 10 ml of 1% BSA diluted in PBS (1x). Incubate the columns overnight to decrease non-specific binding of proteins and thus exosomes to the column.
3. On the following day, remove the 1% BSA and wash manually with 2 ml of PBS (1x). Decant the liquid.
4. Wash the membrane with 10 ml of PBS (1x) with a 10 min centrifugation at 3000 g at room temperature.
5. Conditioned medium (see next section) can be loaded on the membrane at this point.

Preparation and Concentration of iPSC-CMs Conditioned Medium

1. iPSC-CMs are allowed to grow in RPMI/B27+Ins medium for 24–48 hours. Conditioned medium is then collected and can be stored at 4°C for short-term storage. Alternatively, conditioned medium can be frozen at –80°C for long-term storage. *Note: ensure FBS is not used as it contains a significant amount of exosomes, alternatively exosomes should be depleted prior to use.*
2. Centrifuge conditioned medium at 500 g for 5 min at 4°C. Collect the supernatant and discard the pellet.
3. Centrifuge the supernatant at 3000 g for 10 min at 4°C. Collect the supernatant and discard the pellet.
4. Filter the supernatant through a 0.22 µm filter.
5. The filtrated conditioned medium can then be loaded into filter units (see previous section) for concentration.
6. Upon loading of filtrate into filter units, the columns can be concentrated by centrifugation at 3000 g at room temperature. Length of centrifugation and sample volume input will need to be optimized to obtain a desired concentration factor, a 20x-fold concentration is recommended to avoid exosome aggregation, vesicle damage, or loss.
7. The concentrate can then be recovered manually by pipetting and stored at 4°C. It is recommended to continue subsequent processing steps within the next few hours to prevent degradation of exosomal sample.

Isolation of Exosomes

There are various methods for isolation of exosomes, including ultracentrifugation, precipitation-based methods, and immunobeads. Here, we describe a method (Total exosome isolation reagent from cell culture media, ThermoFisher) that only requires general laboratory equipment and hence is broadly applicable to most laboratories.

1. 0.5 volumes of total exosome isolation reagent is added to the concentrated conditioned medium (e.g. 500 μ l of reagent to 1 ml of concentrate). Mix well by vortexing until the solution is homogenous.
2. Incubate samples at 4°C overnight.
3. On the following day, centrifuge the samples at 13000 g for 1 hour at 4°C.
4. Aspirate and discard the supernatant. Care should be taken to prevent accidental dislodging of the pellet (may be invisible at times).
5. Resuspend the pellet in a desired volume of 1x PBS, and the exosomes are now ready for subsequent analysis and can be stored at 4°C for short-term storage or –20°C for long-term storage. A representative image of iPSC-CMs-derived exosomes as characterized by transmission electron microscopy is shown in Figure 1.
6. Alternatively, the pellet can be used immediately for RNA isolation by resuspension in lysis buffer.

RNA Isolation from Exosomes

The authors have routinely used miRCURY™ RNA isolation kit–cell & plant from Exiqon to isolate exosomal RNA.

1. Add 350 μ l of lysis solution with β -mercaptoethanol directly to the exosome pellet and resuspend well.
2. Add 200 μ l of 100% ethanol to the lysate and mix well by vortexing.
3. Assemble a column with provided collection tubes. Apply all the lysate with ethanol onto the column and centrifuge for 1 min at 13000 g.
4. Discard the flowthrough and reassemble the spin column with its collection tube.
5. Add 400 μ l of wash solution to the column and centrifuge for 1 min at 13000 g.
6. Discard the flowthrough and reassemble the spin column with its collection tube.
7. Repeat washing the column 2 more times by adding another 400 μ l of wash solution and centrifuge for 1 min at 13000 g.
8. Discard the flowthrough and reassemble the spin column with its collection tube.
9. Spin the column for 2 min at 13000 g to thoroughly dry the resin. Discard the collection tube.
10. Place the column into a new elution tube. Add 35 μ l of RNase-free water to the column.

11. Centrifuge for 2 min at 200 g, followed by 1 min at 13000 g.
12. The purified RNA sample can now be stored at -80°C for subsequent processing including microRNA analysis and sequencing.

Using this isolation method, the authors have detected microRNAs based on several platforms, including TaqMan assays, SYBR-Green-based microRNA qPCR arrays, and Illumina HiSeq 2000. An electropherogram of exosomal microRNA extracted using the methods above, and analyzed by Bioanalyzer 2100 on a small RNA chip is shown in Figure 2.

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Figure 1. Characterization of iPSC-CMs-derived exosomes by transmission electron microscopy. A round (~120 nm diameter) exosome containing a lipid bilayer is shown (arrowhead). It has an electron-dense content.

Figure 2.

Electropherogram of exosomal small RNAs isolated from iPSC-CM conditioned medium measured by Bioanalyzer 2100 with the Small RNA assay. The electropherogram is plotted as fluorescence intensity (Y-axis) versus size (X-axis). Two distinct regions are defined with the Small RNA assay including small RNA region from 0 to 150 nt (delineated by dashed green lines) and the microRNA region from 10 to 40 nt (delineated by dotted grey lines). Nearly 40% of miRNAs were detected for this particular sample. The data can also be displayed as a densitometry plot as shown on the right.