Published in final edited form as:

Methods Mol Biol. 2017 ; 1660: 389–394. doi:10.1007/978-1-4939-7253-1_32.

Isolation of Extracellular Vesicles from Stem Cells

Zixin Chen^{1,2}, Yongjun Li^{1,3}, Hong Yu⁴, Yan Shen¹, Chengwei Ju^{1,3}, Genshan Ma³, Yutao Liu¹, Il-man Kim¹, Neal L. Weintraub¹, and Yaoliang Tang⁵

¹Vascular Biology Center, Department of Medicine, Medical College of Georgia, Augusta University, 1459 Laney Walker Blvd, CB3303A, Augusta, GA, 30912, USA

²The First Clinical Medical College, Guangzhou University of Chinese Medicine, Guangzhou Shi, Guangdong Sheng, China

³Department of Cardiology, Zhongda Hospital, Medical School of Southeast University, Nanjing, People's Republic of China

⁴Department of Cardiology, Second Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, People's Republic of China

⁵Vascular Biology Center, Department of Medicine, Medical College of Georgia, Augusta University, 1459 Laney Walker Blvd, CB3303A, Augusta, GA, 30912, USA

Abstract

Transplanted induced pluripotent stem cells (IPSC) and embryonic stem cells (ESC) exhibit enhanced survival in ischemic tissues and promote survival of neighboring cells via paracrine effects. Recent studies indicate that stem cells can secrete extracellular vesicles (EV), which can shuttle noncoding RNA between cells and facilitate intercellular signaling and communication between donor stem cells and recipient tissues. Direct transplantation of IPSC-derived EV (IPSC-EV) is highly effective at promoting survival and preventing apoptosis of cardiomyocytes in a mouse model of acute myocardial ischemia–reperfusion (MI/R). Here, we describe a feasible protocol to purify EV from cultured IPSC.

Keywords

Induced pluripotent stem cells; Extracellular vesicles

1 Introduction

Acute myocardial ischemia–reperfusion (MI/R) injury leads to cardiomyocyte necrosis and apoptosis [1]. Because the adult mammalian heart has very limited regenerative capacity, loss of cardiomyocytes leads to scar formation, left ventricular remodeling and heart failure [2]. Recent evidence indicates that the transplantation of stem cells into the heart can promote cardiac repair via paracrine effects [3–7]. Stem cells can secrete extracellular vesicles (EV) [8, 9], which carry noncoding RNAs, including microRNA (miRNA) and long noncoding RNA (lncRNA), which control gene expression via posttranscriptional repression

[10–12] or epigenetic regulation [13]. In stem cells, noncoding RNAs have been shown to play critical roles in modulating cell differentiation, proliferation, and survival [14, 15]. EV play an important role in cell–cell communication via delivering noncoding RNAs. We have reported that EV-mediated paracrine effects contribute for beneficial effect of cardiac progenitor cell transplantation [8]. Recently, we reported that administration of purified IPSC-EV can efficiently protect cardiomyocytes from oxidative stress induced apoptosis in vitro and acute myocardial apoptosis in mouse model of acute MI/R [9].

Numerous methods have been developed to purify EV from the culture media of stem cells, such as ultracentrifugation, Polyethylene Glycol(PEG) based purification, antibody-based purification, et al. Ultracentrifugation method is the traditional method for isolating EV, although PEG purification is a more feasible approach. In this protocol, we describe a PEG4000-based protocol in which a large amount of EV can be purified from small number of cultured IPSC.

2 Materials

2.1 Equipment

1. SW 28 Ti Rotor, Swinging Bucket, Aluminum, 6×38.5 ml, $141,000 \times g$ (Beckman Coulter, 342,204).
2. Centrifuge Tubes, Ultra-Clear™, 38.5 ml, 25×89 mm (Beckman Coulter, 344,058).
3. Optima™ L-90K Ultracentrifuge (Beckman Coulter).
4. Transmission electron microscope (TEM) (JEOL JEM-1230 TEM, Peabody, MA).
5. ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) and corresponding software ZetaView 8.02.28.

2.2 Reagent Setup

1. Normal mouse ES cell culture medium: DMEM high glucose, 15% ES cell qualified FBS, $1 \times$ nonessential amino acids, $1 \times \beta$ -mercaptoethanol, $1 \times$ antibiotic–antimycotic, $1 \times$ L-glutamine and 1000 Units/ml LIF.
2. EV-depleted FBS: Add ES cell qualified FBS equally to six Beckman Coulter Ultra-Clear centrifuge tubes (38.5 ml) with an adapter, then place centrifuge tubes with adapters into an Optima™ L-90K Ultracentrifuge with SW28 Ti Rotor and spin at $120,000 \times g$ for 18 h at 4°C , finally, collect the supernatant of each centrifuge tube, aliquot into 50 ml tubes and store at -20°C .
3. EV depleted mouse ES cell culture medium: DMEM high glucose, 15% EV depleted ES cell qualified FBS, $1 \times$ nonessential amino acids, $1 \times \beta$ -mercaptoethanol, $1 \times$ antibiotic–antimycotic, $1 \times$ L-glutamine, and 1000 Units/ml LIF.

3 Methods

3.1 Cell Culture Using EV Depleted Culture Media

1. Thaw mouse IPSC cells into gelatin coated 100 mm dish, and culture with normal ES cell culture medium (Fig. 1, *see Note 1*).
2. Dissociate mouse IPSC with HyQTase™ and passage in situ every 72 h until 70% confluent.
3. Replace mouse ES cell culture medium with EV depleted mouse ES cell culture medium.
4. Collect supernatant after 48 h, centrifuge at 1000 rpm for 10 min at 4 °C to remove cells.
5. Pass the supernatant through 0.22 µm filters to remove cell debris.

3.2 Purification of EV by Precipitation Using PEG (See Note 2)

1. Transfer 0.22 µm filtered supernatants to a 15 ml centrifuge container and add polyethylene glycol (PEG) 4000 (to final concentration of 8.5%) and NaCl (to final concentration of 0.4 M) to the supernatant and mix well by inverting.
2. Store at 4 °C overnight.
3. Centrifuge the centrifuge container at $1358 \times g$ for 15 min at 4 °C to precipitate EV pellet (white).
4. Remove the supernatant, wash the pellet with 1 ml PBS one time gently.
5. Resuspend the white pellet with PBS (50 µl PBS/ 5×10^6 cells) by vigorous pipetting (*see Note 3*).
6. Aliquot the resuspended EV into 0.2 ml PCR tubes, and store at -80 °C.

3.3 Imaging IPSC-EV Using Transmission Electron Microscopy (TEM)

1. Place 3 µl of PBS containing EVs on formvar carbon-coated 200-mesh copper electron microscopy grids.
2. Incubate for 5 min at room temperature (RT).
3. Perform standard uranyl acetate staining, wash the grid with three exchanges of PBS, and semidry the grid at room temperature.
4. Image the grid using TEM (Fig. 2a, *see Note 4*).

¹It is important to prevent IPSC differentiation under feeder-free culture conditions. We typically add PD0325901 (1 µM final concentration, MEK inhibitor, StemGent, Cat. 04-0006) and CHIR99021 (3 µM final concentration, GSK3 inhibitor, StemGent, 04-0004) to mouse ES cell culture medium to prevent spontaneous differentiation of IPSC.

²EV can also be purified by ultracentrifugation as described in studies of embryonic stem cell- [16] and human CD34+ stem cell-derived EV [17]. Briefly, centrifuge the culture media at $217 \times g$ for 5 min to remove the cells; pass the media through 0.22 µm syringe filters to remove cell debris, and use ultracentrifugation to pellet EV ($100,000 \times g$ for 1 h) on a 30% sucrose-D₂O solution (density ~ 1.127 g/cm³).

³Quantify the cell number to determine the amount of PBS needed to dissolve EV by using HyQTase cell detachment reagent to digest the cultured IPSC into single cells post media harvest.

3.4 Measuring the Size Distribution Using Nanoparticle Tracking Analysis (NTA)

1. Dilute isolated EV samples with 1× PBS buffer to measure the particle size and concentration (Fig. 2b).
2. Record NTA measurement and analyze at 11 positions.
3. Calibrate the ZetaView system using 100 nm polystyrene particles.
4. Measure Zeta potential using 0.05× PBS instead of 1× PBS to adjust conductivity to approximately 500 $\mu\text{S}/\text{cm}$ (*see* Note 5).

Acknowledgments

This work was supported NIH grants AR070029 and HL086555 (to Y.T.), and HL076684 and HL62984 (to N.L.W.).

References

1. Phillips MI, et al. Vigilant vector: heart-specific promoter in an adeno-associated virus vector for cardioprotection. *Hypertension*. 2002; 39(2 Pt 2):651–655. [PubMed: 11882625]
2. Phillips MI, Tang YL, Pinkernell K. Stem cell therapy for heart failure: the science and current progress. *Futur Cardiol*. 2008; 4(3):285–298.
3. Tang YL, et al. Paracrine action enhances the effects of autologous mesenchymal stem cell transplantation on vascular regeneration in rat model of myocardial infarction. *Ann Thorac Surg*. 2005; 80(1):229–236. discussion 236–7. [PubMed: 15975372]
4. Tang YL, et al. Autologous mesenchymal stem cell transplantation induce VEGF and neovascularization in ischemic myocardium. *Regul Pept*. 2004; 117(1):3–10. [PubMed: 14687695]
5. Pan A, Weintraub NL, Tang Y. Enhancing stem cell survival in an ischemic heart by CRISPR-dCas9-based gene regulation. *Med Hypotheses*. 2014; 83(6):702–705. [PubMed: 25459138]
6. Chen L, et al. Two-step protocol for isolation and culture of cardiospheres. *Methods Mol Biol*. 2013; 1036:75–80. [PubMed: 23807787]
7. Tang YL, et al. Cardiac-derived stem cell-based therapy for heart failure: progress and clinical applications. *Exp Biol Med* (May-wood). 2013; 238(3):294–300.
8. Chen L, et al. Cardiac progenitor-derived Exosomes protect ischemic myocardium from acute ischemia/reperfusion injury. *Biochem Biophys Res Commun*. 2013; 431(3):566–571. [PubMed: 23318173]
9. Wang Y, et al. Exosomes/microvesicles from induced pluripotent stem cells deliver cardioprotective miRNAs and prevent cardiomyocyte apoptosis in the ischemic myocardium. *Int J Cardiol*. 2015; 192:61–69. [PubMed: 26000464]
10. Zhang L, et al. MiR-92a regulates viability and angiogenesis of endothelial cells under oxidative stress. *Biochem Biophys Res Commun*. 2014; 446(4):952–958. [PubMed: 24650666]
11. Zhang L, et al. miR-92a inhibits vascular smooth muscle cell apoptosis: role of the MKK4-JNK pathway. *Apoptosis*. 2014; 19(6):975–983. [PubMed: 24705900]
12. Tang Y, et al. MicroRNA-150 protects the mouse heart from ischaemic injury by regulating cell death. *Cardiovasc Res*. 2015; 106(3):387–397. [PubMed: 25824147]

⁴Purified IPSC-EV are sticky and typically resemble a mass of round-shaped cluster of grapes under electron microscopy. Sonication is commonly used to dissociate aggregated EV [18].

⁵The IPSC-EV are not stable and have a tendency to aggregate, which can be measured by zeta potential, a physical property of EV stability. We found that the zeta potential for IPS-EV is -15.44 mV at 23°C . Nanoparticles are stable at absolute zeta potential values ranging from -31 to -40 mV [19]. The measured zeta potential indicates that these EV are not very stable in solution at 23°C . Temperature also affects zeta potential positively; the zeta potential for IPSC-derived exosomes increases to -23.93 mV at 37°C , indicating that EV might become more stable in higher temperature environments [9].

13. Tang Y, et al. Cross talk between the Notch signaling and noncoding RNA on the fate of stem cells. *Prog Mol Biol Transl Sci.* 2012; 111:175–193. [PubMed: 22917231]
14. Archer K, et al. Long non-coding RNAs as master regulators in cardiovascular diseases. *Int J Mol Sci.* 2015; 16(10):23651–23667. [PubMed: 26445043]
15. Bayoumi AS, et al. Crosstalk between long noncoding RNAs and microRNAs in health and disease. *Int J Mol Sci.* 2016; 17(3):356. [PubMed: 26978351]
16. Khan M, et al. Embryonic stem cell-derived exosomes promote endogenous repair mechanisms and enhance cardiac function following myocardial infarction. *Circ Res.* 2015; 117(1):52–64. [PubMed: 25904597]
17. Sahoo S, et al. Exosomes from human CD34(+) stem cells mediate their proangiogenic paracrine activity. *Circ Res.* 2011; 109(7):724–728. [PubMed: 21835908]
18. Ansa-Addo EA, et al. Human plasma membrane-derived vesicles halt proliferation and induce differentiation of THP-1 acute monocytic leukemia cells. *J Immunol.* 2010; 185(9):5236–5246. [PubMed: 20921526]
19. Ostolska I, Wisniewska M. Application of the zeta potential measurements to explanation of colloidal CrO stability mechanism in the presence of the ionic polyamino acids. *Colloid Polym Sci.* 2014; 292(10):2453–2464. [PubMed: 25242857]

Fig. 1. Characterization of mouse induced pluripotent stem cells (IPSC). (a) Mouse IPSC cultured on dishes. (b1–b4) Immunofluorescent staining for the classic stem cell markers SSEA-1 (*green*) and Nanog (*red*); nuclei were counterstained with Draq5 (*blue*)

Fig. 2. Characterization of mouse iPSC-EV. (a) Electron micrograph image of iPSC-derived EV. The image shows a mass of round-shaped vesicles that resemble a cluster of grapes. Scale bar = 50 nm; (b) particle size distribution in purified pellets consistent with size range of EV (average size 100 nm), measured by ZetaView® Particle Tracking Analyzer