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Isolation, characterization and differentiation of mouse cardiac progenitor cells

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Abstract

Despite several strategies developed for replenishing the dead myocardium after myocardial infarction (MI), stem cell therapy remains the leading method to regenerate new myocardium. Although induced pluripotent stem cells (iPS) and trans-differentiation of the differentiated cells have been used as novel approaches for myocardial regeneration, these approaches did not yield very successful results for myocardial regeneration in *in vivo* studies. Asynchronous contractility of newly formed cardiomyocytes with the existing cardiomyocytes is the most important issue with iPS approach, while very low yield of transdifferentiated cardiomyocytes and their chances to beat in the same rhythm as existing cardiomyocytes in the MI heart are important caveats with trans-differentiation approach. CSCs are present in the heart and they have potential to differentiate into myocardial cells. However, the number of resident CSCs are very low. Therefore, it is important to get maximum yield of CSCs during isolation process from the heart. Increasing the number of CSCs and initiating their differentiation *ex vivo* are crucial for CSCs-based stem cell therapy. Here, we present a better method for isolation, characterization and differentiation of CSCs from the mouse heart. We also demonstrated morphological changes in the CSCs after two-days, three-days and seven-days in maintenance medium and a separate group of CSCs cultured for twelve-days in differentiation medium using phase-contrast microscopy. We have used different markers for identification of CSCs isolated from the mouse heart such as a well-established marker for mouse CSC, Sca-1, cardiac specific markers NKX2-5, MEF2C, GATA4, and stemness markers OCT4 and SOX2. To characterize the differentiated CSCs, we used CSCs maintained in differentiation medium for twelve-days. To evaluate differentiation of CSCs, we determined the expression of cardiomyocyte specific markers, actinin and troponin I. Overall; we described an elegant method for isolation, identification, differentiation, and characterization of CSCs from the mouse heart.

Keywords

cardiac stem cells; CSC differentiation; CSC characterization; Sca-1; actinin; troponin I

1. Introduction

Loss of myocardial tissue due to myocardial infarction, diabetes mellitus, or other pathological conditions leads to heart failure, which is the leading cause of morbidity and mortality in the world [1, 2]. Although annual turnover of cardiomyocyte decreases with age, the number of cardiomyocytes remains constant during the human lifespan [3]. It is because of the unique features of the mammalian heart to maintain turnover of cardiomyocytes (by renewal of cardiomyocytes) and microvasculature (smooth muscle and endothelial cells) throughout life [4, 5]. The mammalian heart contains distinct types of endogenous stem and progenitor cells, which have potential for self-renewal, clonogenicity, and multi-lineage differentiation [5–14]. However, the numbers of CSCs or cardiac progenitor cells decreases with ageing [15]. The regenerative approach to increase the number of CSCs for cardiac repair provides a promising strategy for ischemic and/or ageing hearts [16, 17]. For regenerative therapy, it is crucial to characterize CSCs and trace CSCs-derived cardiomyocytes [18–20]. The morphology of cultured CSCs changes with time (Fig. 1). After two days in culture, CSCs increased in number (Fig. 1A). On day three, they started increasing in size (Fig. 1B) and continued increasing in size until day seven (Fig. 1C). CSCs cultured in a differentiation medium for twelve-days showed cardiomyocyte like phenotype (Fig. 1D). At day seven, we stained CSCs with different markers. For stem cells, we used well-validated mouse stem cell marker Sca-1 (Fig. 2A). For cardiac origin, we used NKx2-5 (Fig. 2B), GATA4 (Fig. 2C), and MEF2C (Fig. 2D) markers. We also validated stemness of these cells using pluripotency markers OCT4 (Fig. 2E) and SOX2 (Fig. 2F). To determine whether these CSCs were differentiated into cardiomyocytes, we used CSCs cultured for twelve-days in differentiation medium and determined the expression of cardiomyocyte markers actinin (Fig. 3a) and troponin I (Fig. 3b). The characterization of CSCs and CSCs-derived cardiomyocytes validate our successful isolation of CSCs from the mouse heart.

2. Materials

Mice were ordered from the authorized vendor such as The Jackson Laboratories.

2.1. Mice: C57BL/6J is procured from The Jackson Laboratories, USA.

2.2. Mouse CSCs culture medium components (maintenance and differentiation media, Millipore, USA).

2.3. Mouse cardiac progenitor cells isolation buffer components

- (i) Histopaque solution (Sigma, USA)
- (ii) 1Filter sterilized 1% collagenase I stock solution in HBSS. (0.2% working solution diluted in HBSS) (Sigma, USA)
- (iii) 1X Ham's balance salt solution (HBSS) (Gibco, USA)

2.4. Surgical equipment

- (i) Surgical scissors (Fisher, USA)

- (ii) Fine surgical scissor (Fisher, USA)
- (iii) Curve shank forceps (Fisher, USA)
- (iv) Surgical blade (Hindustan surgical, USA)

2.5. Instruments

- (i) Cell counter (BioRad, USA)
- (ii) Microscope (EVOS, USA)
- (iii) Centrifuge machine (Thermo Scientific, USA)

2.6. Tissue culture

- (i) 10 mm Petri dish (Thermo Scientific, USA)
- (ii) 6-well plate (Thermo Scientific, USA)
- (iii) 24-well plate (Thermo Scientific, USA)
- (iv) T-25 and T-75 culture flask (Thermo Scientific, USA)
- (v) 50 ml and 15 ml conical tubes (Thermo Scientific, USA)
- (vi) 10 µl, 200 µl and 1000 µl pipette tips (Thermo Scientific, USA)
- (vii) 10 µl, 200 µl and 1000 µl pipette man (Eppendorf, USA)
- (viii) Pasture pipette (Fisher, USA)
- (ix) 5 ml, 10 ml, 25 ml Serological disposable pipettes (Thermo Scientific, USA)
- (x) 0.22 µm filter (Fisher, USA)
- (xi) 20 µm and 40 µm sterile cell strainers (Fisher, USA)
- (xii) 10 ml syringe (BD, USA)
- (xiii) Disposable Hemocytometer slide and Trypan Blue dye (BioRad, USA)

2.7. Others

- (i) Powder free nitrile gloves (Fisher, USA)
- (ii) 10X PBS (PH 7.4, cell culture grade) (Lonza, USA)
- (iii) 70% ethanol (Fisher, USA)
- (iv) 10% bleach (disinfectant) (Fisher, USA)

3. Method

3.1 Isolation method of Cardiac progenitor cells

- (i) Ten to fourteen-week old, four or five adult mice were euthanized using CO₂ chamber.
- (ii) Mice were sterilized with 70% ethanol under the hood.

- (iii) Thoracic cavity of each mouse was opened inside the hood, washed with cold PBS, and blood was removed.
- (iv) The mouse heart was surgically removed and placed into a sterile 10 cm petri dish containing 10 ml of cold 1X PBS.
- (v) The heart was gently palpitated using curved shanked forceps to remove the blood left inside the heart. Atrium of the heart is dissected out and ventricles were used for CSCs isolation.
- (vi) The extracted ventricles was transfer onto a 50 ml conical tube containing 10 ml chilled Hams' balance salt solution (HBSS) on ice and kept until further processing (*see* Note 1).
- (vii) The ventricles containing tube was placed in a Biosafety cabinet for further processing (*see* Note 2).
- (viii) Under the Biosafety cabinet, the ventricles were transferred into a sterile 10 cm petri dish containing 5-10 ml cold HBSS.
- (ix) Ventricles were mince into very small pieces using fine surgical scissor (*see* Note 3).
- (x) Ventricular mince tissue was centrifuged at 500g at 4°C for 5 min and supernatant was removed.
- (xi) The pellet from the previous step was transferred into a 50 ml conical tube.
- (xii) 5-6 ml 0.2% collagenase solution prepared in HBSS was added to the pellet (*see* Note 4).
- (xiii) The pellet was mixed thoroughly with the collagenase solution by agitating the tube or rocking the tubes on a rocker shaker for 60-90 minutes at 37°C at 150 rpms (*see* Note 5).
- (xiv) Using either wide orifice 1000 µl pipette or cut 1000 µl narrow orifice pipette tip, the ventricle tissue pellet was triturated for proper dissociation of the pellet (*see* Note 6).
- (xv) The lysis process of collagenase was then stopped by adding cardiac stem cells maintenance medium (*see* Note 7).
- (xvi) Filtered the cell suspension through 100µm cell strainer to remove the larger undigested tissue pieces.

¹.Replace the HBSS after each dissection to ensure almost complete clearance of blood.

².Make Biosafety cabinet completely sterile using 70% ethanol before placing the ventricles. Wipe the tubes with 70% ethanol before placing it in the Biosafety cabinet.

³.Ventricles were hold tight by forceps and cut into small pieces (1-2 mm in diameter) using scissor or surgical blade.

⁴.The volume of the 0.2% collagenase solution should be double of the volume of the pellet.

⁵.Shake the tube vigorously with hand every 30 min to enhance the lysis.

⁶.To get the maximum recovery of cells, we should triturate the large pieces at least 3-5 min.

⁷.The volume of the maintenance medium should be two- three times the volume of the collagenase.

- (xvii) Further, passed filtrate through 40µm cell strainer to remove any endothelial cells.
- (xviii) Gently overlaid the equal volume of filtrate over histopaque solution. For example, in a 50 ml tube, add 20 ml histopaque solution first and then add 20 ml filtrate solution over the histopaque solution (*see Note 8*).
- (xix) Centrifuged the tube containing filtrate and histopaque solution in a swing bucket centrifuge at 500g for 20 min at RT (*see Note 9*).
- (xx) After centrifugation, the tube was carefully taken out. The tube had three layers: upper medium layer, middle buffy coat-containing CSCs and lower histopaque layer containing mixed cell population.
- (xxi) Using 1 ml pipette, the upper and the middle layers were transferred into fresh 15 ml conical tube (*see Note 10*).
- (xxii) An equal volume of pre-warm CSCs maintenance medium was added to the cells suspension and it was mixed properly.
- (xxiii) The tube containing the CSCs with the CSCs maintenance medium was centrifuged at 500g/5 min/ 4°C. The supernatant was discarded and the pellet containing CSCs were washed with incomplete DMEM medium to remove residual histopaque. Finally, the purified CSCs pellet was collected.
- (xxiv) The CSCs containing pellet was re-suspend in 1 ml cardiac stem cell maintenance medium.
- (xxv) These CSCs were then seeded into a culture plate (*See Note 11*). They were cultured into cell culture incubator maintained at 37°C with 5% CO₂.
- (xxvi) Change the medium of cells after every day for at least three days with fresh pre-warm cardiac stem cells maintenance medium. After that, change the medium of the cells every alternate day.
- (xxvii) Observe the cells morphology and growth condition under the microscope and capture images of the CSCs.

3.2. Maintenance of cardiac stem cells

- (i) After reaching confluent growth of the cells in flask, transfer cells from one T-25 flask into a new T-25 flask by trypsinization. At this stage, cells are considered as passage 1 (P1).

⁸It is a very important step of the protocol where care should be taken to avoid mixing of the two solutions. For that, the filtrate solution should be added very slowly onto the histopaque solution in a tube.

⁹Keep the setting of centrifuge with lowest acceleration/deceleration speed to keep proper separation and to avoid mixing of the two solutions.

¹⁰Take precaution during removal of buffy layer. Avoid sucking histopaque layer because histopaque solution is toxic to the CSCs.

¹¹Depending on CSCs number/density, they will be cultured in six-well plate or T25 flask. For low density six-well plate is better. Cells number can be determined by using Hemocytometer.

- (ii) The confluent P1 cells was passaged into two T-25 flask (P2). At this stage, cells either can be propagated for experimentation by transferring then into a T-75 flask or can be stored into liquid nitrogen for future experiments.
- (iii) To maintain the cells in the culture medium, one million CSCs were seeded in a T-25 flask or two million CSCs were seeded in a T-75 flask (*See* Note 12).

3.3. Characterizations of isolated cardiac progenitor cells

To characterize isolated CSCs

- (i) Take phase contrast images under light microscope. Initial cell morphology will look like spindle shape or mesenchymal stem cell like morphology (Figs. 1A-B).
- (ii) Further, stain the cells with immunofluorescence antibodies for detection of CSCs markers Sca-1, cardiac markers NKx2.5, GATA4, MEF2C, and pluripotency markers OCT4 and SOX2 (Figs. 2A-F).
- (iii) For fluorescent staining of CSCs, seed 10,000 cells in a 24-well culture plate.
- (iv) After one day, fix the cells with 4% formalin solution for 15 min.
- (v) After fixation, wash the cells with cold PBS for 5 min, three times.
- (vi) Permeabilized the cells with 0.2% Triton-X-100 in PBS for 10 min (*See* Note 13).
- (vii) Wash the cells with cold PBS for 5 min, three times.
- (viii) Block the cells with 1% BSA in PBST (PBS+ 0.1% Tween 20) for 1 hr.
- (ix) After the blocking, incubate the cells with primary antibody diluted in 0.5% blocking solution for overnight (*See* Note 14).
- (x) After overnight primary antibody incubation, wash the cells with cold PBS with 5 min, three times.
- (xi) Incubate the cells with secondary antibody diluted in 0.5% blocking solution for 1 hr. (*See* Note 15).
- (xii) After competition of incubation, remove the secondary antibody solution and washed the cells with cold PBS for 5 min, three times.
- (xiii) Counter stain the cells with dapi, 1 µg/ml for 5 min.
- (xiv) Capture the images using fluorescent microscope.
- (xv) Keep the plate in dark at 4°C.

¹²It is necessary to passage CSCs every three to four days or when they attain 85% confluency. Cells can be maintained in CSCs maintenance medium for up to three to four weeks.

¹³No need of permeabilization if detecting surface antigen or receptor.

¹⁴Dilution of antibodies should make as per data sheet specification for immunocytochemistry.

¹⁵Dilution of secondary antibodies should be as per primary antibody dilution.

3.4. Differentiation of CSCs into cardiomyocyte

For differentiation of CSCs

- (i) Seed 50,000 cells in a 6-well plate in CSCs maintenance medium.
- (ii) At 85-90% confluency, replace the medium with CSCs differentiation medium and culture these cells into cell culture incubator for 2-3 weeks (*See Note 16*).
- (iii) Replace the medium after every 2-3 days.
- (iv) After 2-3 weeks of differentiation, stain the differentiated cells with cardiomyocyte markers such as Troponin I and Actinin (Figs 3A–3B).

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References

- Lloyd-Jones D, et al., Executive summary: heart disease and stroke statistics--2010 update: a report from the American Heart Association. *Circulation*, 2010 121(7): p. 948–54. [PubMed: 20177011]
- Schnell O, et al., Type 1 diabetes and cardiovascular disease. *Cardiovasc Diabetol*, 2013 12: p. 156. [PubMed: 24165454]
- Bergmann O, et al., Dynamics of Cell Generation and Turnover in the Human Heart. *Cell*, 2015 161(7): p. 1566–75. [PubMed: 26073943]
- Bergmann O, et al., Evidence for cardiomyocyte renewal in humans. *Science*, 2009 324(5923): p. 98–102. [PubMed: 19342590]
- Monsanto MM, et al., Concurrent Isolation of 3 Distinct Cardiac Stem Cell Populations From a Single Human Heart Biopsy. *Circ Res*, 2017 121(2): p. 113–124. [PubMed: 28446444]
- Beltrami AP, et al., Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell*, 2003 114(6): p. 763–76. [PubMed: 14505575]
- Ellison GM, et al., Adult c-kit(pos) cardiac stem cells are necessary and sufficient for functional cardiac regeneration and repair. *Cell*, 2013 154(4): p. 827–42. [PubMed: 23953114]
- Ellison GM, et al., Endogenous cardiac stem cell activation by insulin-like growth factor-1/hepatocyte growth factor intracoronary injection fosters survival and regeneration of the infarcted pig heart. *J Am Coll Cardiol*, 2011 58(9): p. 977–86. [PubMed: 21723061]
- Hou X, et al., Isolation, Characterization, and Spatial Distribution of Cardiac Progenitor Cells in the Sheep Heart. *J Clin Exp Cardiol*, 2012 S6.
- Chong JJ, et al., Adult cardiac-resident MSC-like stem cells with a proepicardial origin. *Cell Stem Cell*, 2011 9(6): p. 527–40. [PubMed: 22136928]
- Bu L, et al., Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages. *Nature*, 2009 460(7251): p. 113–7. [PubMed: 19571884]
- Smits AM, et al., Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes: an in vitro model for studying human cardiac physiology and pathophysiology. *Nat Protoc*, 2009 4(2): p. 232–43. [PubMed: 19197267]
- Ellison GM, Nadal-Ginard B, and Torella D, Optimizing cardiac repair and regeneration through activation of the endogenous cardiac stem cell compartment. *J Cardiovasc Transl Res*, 2012 5(5): p. 667–77. [PubMed: 22688972]
- Oh H, et al., Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. *Proc Natl Acad Sci U S A*, 2003 100(21): p. 12313–8. [PubMed: 14530411]

¹⁶ Always pre-warm the medium before adding to the cells.

15. Laugwitz KL, et al., Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. *Nature*, 2005 433(7026): p. 647–53. [PubMed: 15703750]
16. Soonpaa MH, et al., Formation of nascent intercalated disks between grafted fetal cardiomyocytes and host myocardium. *Science*, 1994 264(5155): p. 98–101. [PubMed: 8140423]
17. Sekine H, et al., Endothelial cell coculture within tissue-engineered cardiomyocyte sheets enhances neovascularization and improves cardiac function of ischemic hearts. *Circulation*, 2008 118(14 Suppl): p. S145–52. [PubMed: 18824746]
18. Wu SM, Chien KR, and Mummery C, Origins and fates of cardiovascular progenitor cells. *Cell*, 2008 132(4): p. 537–43. [PubMed: 18295570]
19. Martin-Puig S, Wang Z, and Chien KR, Lives of a heart cell: tracing the origins of cardiac progenitors. *Cell Stem Cell*, 2008 2(4): p. 320–31. [PubMed: 18397752]
20. Matsuura K, et al., Cell sheet transplantation for heart tissue repair. *J Control Release*, 2013 169(3): p. 336–40. [PubMed: 23500057]

Figure 1.

Phase-contrast images (10× or 100 times and 20× or 200 times magnifications) of isolated mice cardiac stem cells (CSCs) at different days of culture and after 12 days of differentiation. **A1 and A2.** CSCs after two-days of culture in CSC maintenance medium after isolation. **B1 and B2.** CSCs after three-days of culture in CSC maintenance medium after isolation. **C1 and C2.** CSCs after seven-days of culture in CSC maintenance medium after isolation. **D1 and D2.** Cardiomyocyte like cell morphology after twelve days culture of CSCs in CSCs differentiation medium. Scar bar is 400μm for 10× and 200 μm for 20×.

Figure 2.

Immunofluorescence images of mice CSCs stained with different cardiac stem cells and pluripotent stem cells specific markers. Progenitor cells were stained with cardiac stem cells specific markers such as Sca-1, NKx2.5, MEF2C and GATA4. These cells were also positive for pluripotent stem cells markers OCT4 and SOX2. Blue color Dapi is used to stain the nucleus. All the representative images are merged image of specific markers and Dapi. Scale bar is 100µm.

Fig.3. Immunofluorescence images of cardiomyocyte like cells after twelve days of CSCs differentiation. Cells were immunostained with cardiomyocyte specific markers Actinin (A) and Troponin I. (A). Blue color Dapi is used to stain the nucleus. All the representative images are merged image of cardiomyocyte markers with Dapi. Scar bar is 100µm.