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Two-Step Protocol for Isolation and Culture of Cardiospheres

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Abstract

Cardiac progenitor cells (CPC) are a unique pool of progenitor cells residing in the heart that play an important role in cardiac homeostasis and physiological cardiovascular cell turnover during acute myocardial infarction (MI). Transplanting CPC into the heart has shown promise in two recent clinical trials of cardiac repair (SCIPIO & CADUCEUS). CSCs were originally isolated directly from enzymatically digested hearts followed by cell sorting using stem cell markers. However, long exposure to enzymatic digestion can affect the integrity of stem cell markers on the cell surface and also compromise stem cell function. Here, we describe a two-step procedure in which a large number of intact cardiac progenitor cells can be purified from small amount of heart tissue.

Keywords

Cardiac progenitor cells; Cardiosphere; Magnetic-activated cell sorting (MACS)

1 Introduction

The mammalian heart has long been considered a postmitotic organ, with little regenerative capacity following ischemic necrosis, thereby leading to scarring. Recent evidence indicates that the adult mammalian heart (including the human heart) possesses a pool of intermediate cardiac progenitors which can differentiate into cells that phenotypically resemble cardiomyocytes, endothelial cells, and vascular smooth muscle cells, the major components of myocardial tissue [1, 2]. Unfortunately, resident mammalian CPC cell populations become acutely depleted following MI [3], which limits their endogenous regenerative capacity. A recent clinical trial from Cedars-Sinai Heart Institute and Johns Hopkins University demonstrated that transplanting autologous CPC can increase cardiac muscle mass and reduce scar size [4]. Conventional methods use enzymatic digestion to isolate resident cardiac progenitor cells (c-kit+, Sca-1+) from heart in one step. However, enzymatic digestion of myocardium compromises the integrity of important surface antigens of resident cardiac stem cells and leads to dysfunctional sorted cells, which makes the method hard to be reproduced.

To avoid these problems, we have developed a two-step procedure to isolate and expand pure population of cardiac progenitor cells from heart tissue. First, we use primary heart tissue explanting technology to expand endogenous cardiac progenitor cells for about 2 to 3 weeks depending on age; second, we use magnetic- activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) to isolate pure population of progenitor cells from cardiac explant tissue and cloning. We use a cardiac fibroblast conditional medium to maintain the proliferation of cardiac progenitor cells at first three generations. We demonstrated that Sca-1+ cells isolated by this method keep their self-renewal and clonogenic character in vitro and can directly differentiate into normal cells in myocardium,

including cardiomyocytes, endothelial cells, and smooth muscle cells, after transplantation in ischemic hearts of mice.

In this protocol, we describe a two-step procedure in which a large number of Sca-1+ cardiac progenitor cells can be purified from small amount of heart tissue. The cultured cardiac progenitor cells can keep their capacity for self-renewal and clonogenic in vitro with fibroblast-free conditional medium.

2 Materials

2.1 Cell Isolation and Expansion

1. Heart tissue.
2. Dulbecco's PBS (D-PBS, without calcium chloride and magnesium chloride).
3. Bovine serum albumin (BSA, Fraction V, 7.5 % (wt/vol)) solution.
4. Collagenase IV.
5. 24-well plate, with lid, flat bottom, ultralow attachment surface.
6. Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 nutrient broth (1:1, vol/vol) with 15 mM HEPES buffer, L-glutamine, and pyridoxine hydrochloride.
7. Fetal bovine serum (FBS).
8. Human fibroblastic growth factor, basic (rh-bFGF).
9. Trypsin/EDTA [0.05 % (wt/vol)].
10. Penicillin/streptomycin/amphotericin B.
11. Cryogenic vials.
12. Dimethylsulfoxide Hybri-Max.
13. Freezing container (1 °C)
14. 40-µm cell strainer.
15. Fibronectin-coated dish.
16. Poly-D-lysine-coated dish.

2.2 MACS Sorting

1. Anti-Sca-1 microbeads.
2. Magnetic cell sorter device.

2.3 Equipment

1. Centrifuge water bath shaker.

2.4 Reagent Setup

1. Enzyme mix: prepare fresh a solution of 0.2 % (wt/vol) trypsin and 0.1 % (wt/vol) collagenase IV in D-PBS, usually 30–50 ml for each mouse heart.
2. Explant medium: Iscove's Modified Dulbecco's IMDM with 10 % fetal calf serum (FBS), 100 U/mL penicillin G, 100 µg/ml streptomycin, 2 mmol/L L-glutamine, and 0.1 mmol/L 2-mercaptoethanol.

3. Cell growth medium (CGM): [DMEM/F12, 10 % FBS, 200 mmol/L L-glutamine, 0.1 mmol/L β -mercaptoethanol, 1 % nonessential amino acids, 1,000 U/ml LIF, 0.1 U/ml thrombin, and 5 ng/ml basic fibroblast growth factor (bFGF)].
4. Cardiac fibroblast conditional medium (CFCM): CGM was conditioned by exposing it for 48 h to cardiac fibroblast in 10-cm dish to dissolve paracrine factors from cardiac fibro-blasts. The conditional medium will be mixed with fresh CGM medium at a 3:1 ratio, sterilized by filtration, and supplemented with additional bFGF at 5 ng/ml.

3 Methods

3.1 Step One: Dissociating and the Primary Tissue Explant

1. Mince mouse heart tissues into small $1 \times 1 \text{ mm}^3$ pieces in a 10-cm dish.
2. Transfer the minced heart tissues to 10 ml enzyme mix in 50-ml centrifuge tube for enzymatic dissociation.
3. Place the tube in the 37 °C shaker water bath and gently shake (80 rpm/min) for 5 min. Aspirate off the supernatants; leave pellets of minced heart tissues. Add 10 ml new enzyme mix, and repeat Subheading 3.1 steps 2–4 for another two times.
4. Aspirate the supernatants, and resuspend each remaining tissue fragments in 3 ml explant medium and pool the suspensions into 10-cm fibronectin-coated dish at 37 °C and 5 % CO_2 .
5. Add 7 ml explant medium 2 days after tissue explanting, change medium every 3 days, and note the date of a layer of fibroblasts covering the culture dish, and note the date of round, phase-bright cells with different sizes migrating from the adherent explants. These migrated cardiac cells are loosely attached to the fibroblast layer and could be collected periodically by simply washing and centrifuging (*see Note 1*).

3.2 Step Two: The Cell Enrichment

1. Collect small, phase-bright cells migrated from heart explants by washing with D-PBS at 2–3 weeks after tissue explanting (*see Note 2*).
2. Filter the cell suspension through a 40- μm cell strainer, wash the filter with 10 ml of D-PBS, centrifuge the filtered cells at $300 \times g$ for 10 min at RT, and count the cell numbers.
3. Deplete mature hematopoietic cells and their committed precursors from the migrated cardiac cells using Lineage Cell Depletion Kit (mouse).
 - (a) Resuspend cell pellet in 40 μL of buffer, add 10 μL of Biotin-Antibody Cocktail, mix well and incubate for 10 min at 4–8 °C, and add 30 μL of buffer to cells.
 - (b) Add 20 μL of Anti-Biotin MicroBeads to cells. Mix well and incubate for additional 15 min at 4–8 °C. Wash cells by adding 1 mL of buffer and centrifuge at $300 \times g$ for 10 min. Pipette off supernatant completely. Resuspend cells in 500 μL of buffer.

¹ During **step 1** preparation, a layer of fibroblasts will cover the culture dish at about 7 days, and then the round, phase-bright cells with different size will migrate from the adherent explants.

²The round, phase-bright cells migrated from cardiac explants are loosely attached to the fibroblast layer and could be collected periodically by simply washing and centrifuging without using trypsin.

- (c) Magnetic separation with MS Columns, apply cell suspension onto the column, allow the cells to pass through, and collect effluent as fraction with unlabeled cells, representing the enriched lineage negative cell fraction.
- 4. Purify the Sca-1+ cardiac progenitor cells from lineage negative cells using anti-Sca-1-microbead kit from Miltenyi Biotec.
 - (d) Resuspend cell pellet in 90 μ L of buffer, add 10 μ L of Biotinylated Anti-Sca-1 antibody, mix well, and incubate for 10 min at 4–8 °C. Wash cells by adding 1 mL of buffer. Centrifuge at 300 $\times$ *g* for 10 min. Aspirate supernatant completely.
 - (e) Resuspend cell pellet in 80 μ L of buffer; add 20 μ L Anti-Biotin MicroBeads. Mix well and incubate for 10 min at 4–8 °C. Wash cells by adding 1 mL of buffer. Resuspend cells in 500 μ L of buffer.
 - (f) Apply cell suspension onto MS column, wash three times, and remove column from the separator and place it on a suitable collection tube; pipette 1 ml of CFCM medium onto the column. Immediately flush out fraction with the magnetically labeled cardiac cells by firmly applying the plunger supplied with the column.
- 5. Seed newly isolated cardiac Sca-1+ cells at 6 well plates pre-coated with Matrigel using CFCM medium for first three passages (*see* Note 3 and ⁴).
- 6. After cell sorting via MACS, purified Sca-1+ cells were cultured in CFCM (*see* Note 5) in poly-D-lysine-coated dishes. Cardiospheres form after 4–7 days on poly-D-lysine-coated dishes (Fig. 1c).
- 7. Formed cardiospheres can be plated on fibronectin-coated dishes to become a monolayer (cardiosphere-derived cells) (Fig. 1d) for cell transplantation.

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³Cardiac fibroblasts provide a feeder layer for cardiac progenitor cell proliferation and migration from explants. The paracrine factors released from cardiac fibroblasts are important for CPC maintenance and self-renewal. We use CFCM medium which includes conditioned medium from cardiac fibroblast to support cardiac stem cell maintenance and find that CFCM is sufficient to maintain Sca-1+ cells in a proliferative state without the need for fibroblasts.

⁴Extracellular matrix (ECM) is also important for purified CPC maintenance. We found the Matrigel can provide the required ECM for survival and proliferation of purified CPC since it contains laminin, collagen IV, and heparin sulfate proteoglycan.

⁵Sca-1+ cells were bright, round without fibroblast contamination. Some of them proliferated and became 2- to 3-cell aggregates in suspension after 3–5 days. These aggregates slowly increased in size and gradually attached to the plate. After 2 weeks in poly-D-lysine-coated dish, the Sca-1+ cells form three-dimensional spheres (Fig. 1c).

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Fig. 1. Isolation, expansion of cardiosphere-derived cells (CDCs). (a) Explanted minced heart tissue. (b) Round, phase-bright cells migrating from the primary culture of mouse ventricular explants, (c) cardiosphere formation from purified Sca-1 positive cardiac progenitors in poly-D-lysine-coated dish, (d) monolayer expansion of cardiosphere-derived cells in fibronectin-coated dish