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Identification of Stem Cells After Transplantation

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Abstract

The ability to identify the donor stem cells following transplantation into injured hearts is critical. This is particularly important in evaluating stem cell survival and lineage differentiation into mature cardiovascular cells. Several approaches have been employed for tracking the donor stem cells, including fluorescent dyes and fluorescent protein gene transfer. Here, we will induce a protocol using lentivirus-mediated green fluorescent protein (GFP) to monitor the fate of donor stem cells following transplantation.

Keywords

Green fluorescent protein (GFP); Lentivirus; Stem cells; Myocardial infarction (MI)

1 Introduction

Ischemic heart disease is the leading cause of morbidity and mortality in the western world. Endogenous cardiac stem cells are rapidly exhausted after myocardial ischemia [1]; therefore, transplantation with ex vivo expansion stem cells may replenish the pool of stem cells in injured heart and lead to heart regeneration. Identifying the engrafted cells will help us to compare the efficacy of different source of cells and identify the relative mechanisms for heart repair. A number of approaches have been employed to tracking the engrafted stem cells in vivo, for example, (1) fluorescent dye labeling, like PKH26 [2] and DAPI [3]—the limitation of dye labeling is that the observed signal may not come from donor cells but from host cells which have acquired free dye released from dead donor cells; in addition, this approach is transient and suffered from signal dilution—and (2) reporter gene transfer, like GFP [4], *beta-galactosidase* (-gal) [5, 6], and luciferase [7]. Lentiviral vector can infect divided and nondivided cells [8]; it allows monitoring of the fate of donor cells for long term since the reporter genes can be integrated into chromosome [9, 10]. Stem cells from genetically modified animals, for example, GFP mice, provide an alternative strategy to identify donor cells following cell transplantation. In this chapter, we will introduce a detailed protocol to use lentiviral GFP vector to label donor stem cells and identify engrafted cells in ischemic myocardium.

2 Materials

2.1 Plasmids for Lentivirus Packaging

1. pRRLSIN.cPPT.PGK-GFP.WPRE.
2. psPAX2.
3. pMD2.G.
4. 293FT cells.

5. FuGENE® HD Transfection Reagent.
6. 0.45 µm syringe filter.
7. Ultracentrifuge and rotor.
8. Ultra-Clear ultracentrifuge tubes.
9. OPTI-MEM® Reduced Serum Medium.
10. Dulbecco's Modified Eagle Medium (DMEM).
11. Fetal bovine serum (FBS).
12. Hexadimethrine bromide (polybrene).
13. Prepare a 1 mg/mL solution of polybrene in 0.9 % NaCl, and then filter-sterilize through 0.2 µm filter. Stock solution stored at 4 °C.
14. Small animal ventilator.
15. Accutase cell detachment solution.
16. Abbocath-T I.V. 18 G catheter.
17. BD 3/10 ml SafetyGlide Insulin Syringe with 31G needle.
18. Vectashield Hardset mounting medium with DAPI vector.
19. Leica TCS confocal microscope.

3 Methods

3.1 Lentiviral-GFP-Vector Packaging (See Note 1)

Viral vectors encoding GFP were produced by transfection of 293FT cells with the lentiviral backbone plasmid (pRRLSIN.cPPT.PGK-GFP.WPRE plasmid), an envelope plasmid (pMD2.G), and a packaging plasmid (psPAX2).

This protocol is for transfection in a 15-cm dish.

Day 1: Plate 1.2×10^7 293 FT cells in 20 mL of media in a 10-cm tissue culture dish. Incubate cells at 37 °C, 5 % CO₂ overnight.

Day 2:

1. In tube 1, create a mix of plasmids.
 - 6.3 µg pRRLSIN.cPPT.PGK-GFP plasmid.
 - 4.7 µg psPAX2 packaging plasmid.
 - 1.6 µg pMD2.G envelope plasmid to 125 µl OPTI-MEM.
2. In tube 2, create a master mix of FuGENE® HD transfection reagent in OPTI-MEM. Mix 38 µL FuGENE HD + 462 µL OPTI-MEM for each 15 dish transfection (*see* Note 2).
3. Pipette 500 µL of FuGENE HD master mix to tube 1. Mix by gently flicking the tube. Incubate for 30 min at room temperature.

¹ Before this step, you must contact your institution's biosafety office to receive permission and institution-specific instructions. You must follow safety procedures and work in a BL2+ environment suitable for handling HIV-derivative viruses.

² Directly add FuGENE HD into the OPTI-MEM; avoid FuGENE HD in contact with the walls of the polypropylene tube before it has been diluted.

4. Gently add DNA:FuGENE[®] mix dropwise to 293FT. Swirl to disperse mixture evenly (*see Note 3*).
5. Incubate cells at 37 °C, 5 % CO₂ overnight.

Day 3: In the morning, change the media (*see Note 4*). Incubate cells at 37 °C, 5 % CO₂ for 24 h.

Day 4 and Day 5: Harvest supernatant containing lentiviral vector and transfer to 50 ml polypropylene storage tube. Filter the media through a 0.45-µm filter to remove 293FT cells (*see Note 5*). Add ~37 ml of viral supernatant to each ultracentrifuge tube and balance, and then spin at ~82,000 × *g* for 90 min at 4 °C in SW28 rotor; resuspend viral pellet in 400 µl of PBS. Virus should be frozen at –80 °C for long-term storage (*see Note 6*).

3.2 Stem Cell GFP Labeling

Lentivirus can efficiently transduce a broad range of cell types, including both dividing and nondividing cells.

Day 1: Plate 0.3×10^6 stem cells to 6-well plate and incubate at 37 °C, 5 % CO₂ overnight.

Day 2: Stem cells should be approximately 70 % confluent before infection. Change to fresh culture media containing 8 µg/mL polybrene. Add 100 µl lentiviral solution (*see Note 7*). Incubate cells at 37 °C, 5 % CO₂ for 3 days.

Day 5: Change to fresh media, and determine the transduction efficacy using fluorescent microscopy (Fig. 1).

3.3 Myocardial Infarction Model and Cell Transplantation

1. Remove hair from chest region using hair clippers. After anesthesia, surgical preparation, and preemptive analgesics, mice will be intubated with an 18-gauge catheter and mechanically ventilated at 120 breaths per minute with a stroke volume of 150 µl on a positive pressure ventilator with room air. Body temperature will be maintained with a heated water blanket.
2. Make lateral incision to open the chest under the dissecting microscope; cut the intercostals muscle between left 3rd and 4th ribs with a cautery pen. Tear the pericardium to make a small opening, identify the left anterior descending coronary artery (LAD), and pass 8-0 suture under 2–3 mm from the tip of the left auricle. Permanently occlude the LAD by ligation (*see Note 8*).
3. Harvest the GFP-labeled stem cells using Accutase; inactivate Accutase with MEM complete medium. Count cells using a hemacytometer; centrifuge at $300 \times g$ for 5 min. Carefully aspirate supernatant and resuspend pellet at a concentration of 5×10^5 cells/30 µl of PBS for cell transplantation; keep cells in ice prior to injection.
4. Intramyocardial injection: identify the border zone of the heart; make two 15 µL injections of GFP cell suspension using a 31-gauge needle (*see Note 9*).

³ 293FT cells should be 70 % confluent and in DMEM in 10 % FBS without antibiotics.

⁴ Be cautious not to disturb the transfected cells when pipetting the medium.

⁵ Do not use a 0.2-µm filter since it may shear the envelope of lentiviral particles.

⁶ Avoid repeated freeze/thaw cycles since they will decrease the efficiency of the virus.

⁷ Polybrene can significantly increase the efficiency of viral infection; however, it is toxic to cell. To sensitive stem cells, substitute protamine sulfate for polybrene. It is recommended using high MOI (multiplicity of infection) for stem cell infection to reach high transduction efficiency and bright GFP signal.

⁸ Successful coronary occlusion will be verified by the development of a pale color in the distal myocardium after ligation.

⁹ Bending the needle head ~45° can avoid the injection of cells into left ventricular chamber.

5. Following injection, use 6-0 absorbable sutures to close the ribs and muscle layers. Suture skin closed using 6-0 nylon suture also. Allow mouse to recover from anesthesia and extubate.

3.4 Identify Donor Stem Cells in Infarct Heart

1. Five days after surgically induced MI and intramyocardial injection of GFP stem cells, harvest mouse hearts, embed it in OCT compound, snap frozen, and cut into 5- μ m sections.
2. Identify the donor cells, and analyze stem cell differentiation by immunostaining:
 - a. Fix with 4 % formaldehyde for 10 min at room temperature.
 - b. Wash slides twice in PBS for 5 min $\times$ 2.
 - c. Mount slides in DAPI mounting medium.
 - d. Examine the sections under confocal microscope; identify the GFP-positive cells (Fig. 2).

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Fig. 1. Transduced cardiac stem cells with GFP lentivirus. (a) Phase contrast picture of stem cell clone; (b) GFP expression in transduced stem cells

Fig. 2.
Confocal imaging of donor cells in infarcted mouse heart 5 days after surgical MI and cell injection