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Adenovirus-Mediated Gene Transfection in the Isolated Lymphatic Vessels

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Abstract

The authors describe technical details of experimental protocol of gene transfection in isolated rat mesenteric lymphatic vessels (MLVs). Authors also refer to the recent publication in *Microcirculation*, which provides wide set of experimental evidences obtained from confocal microscopic imaging and isolated vessels functional tests, which confirmed a successful achievement of the following goals. (1) Optimization of the experimental conditions to maintain the isolated “normal” rat mesenteric vessels in culture for sufficiently long periods of time to permit effective knockdown or overexpression of selected proteins/genes. (2) Development of the effective transfection protocols for lymphatic muscle and/or endothelial cells in intact isolated rat MLVs without nonspecific impairment on lymphatic contractile function due to the transfection protocol per se.

Keywords

Lymphatic vessel; Adenoviral transfection

1. Introduction

Modern molecular biology offers a variety of novel experimental techniques to influence genes of interest in different subjects of biological research. In some situations when time, resources, and unique tissue properties allow such, researchers may approach the possibility to target genes in freshly isolated tissue specimens which remains their structural integrity and in situ phenotype. Such experimental approach could widen the ability of investigators to focus on the direct effects of gene modification on isolated tissue specimen, with the characteristic that initial organ morphology and function are still intact or only minimally altered, while the experimental conditions are comparatively stable and easy achievable for their permanent control during whole duration of the transfection experiment.

Recently, we developed experimental approaches and protocols to check gene function over the course of up to 2 weeks in lymphatic muscle and/or endothelium from isolated rat lymphatic vessels (1). Technically, rat mesenteric lymphatic vessels (MLV) are particular example of isolated vessels well-suited for gene transfection since the muscle layer is only one to two cells thick, vessel wall only contains one layer of endothelial cells, and the interstitial structures are very thin. Therefore, the experimental protocol presented here is suitable for gene transfection experiments in any isolated blood or lymphatic vessels, which

are comparable by size (~100–150 μm in diameter) and wall structure to the vessels of our choice—rat MLV. To develop the techniques of gene transfections in isolated rat MLV, we focused on two major tasks: (1) Optimize the experimental conditions to maintain the isolated “normal” rat MLV in culture for sufficiently long periods of time to permit effective knockdown or overexpression of selected proteins/genes. (2) Develop effective transfection protocols for lymphatic muscle and/or endothelial cells in intact isolated rat MLV without nonspecific impairment on lymphatic contractile function due to the transfection protocol per se. In our recent manuscript in *Microcirculation* (1), we provided wide set of experimental evidences obtained from confocal microscopic imaging and isolated vessels functional tests. These data confirmed that we successfully achieved our goals stated above. In the present manuscript, we describe more technical details of experimental protocol of gene transfection in isolated rat MLV with belief that our experience may serve in future as a good starting point for further development of such molecular tool.

2. Material

1. Dulbecco's phosphate-buffered saline (DPBS) (Invitrogen Corp.).
2. D-MEM/F12 (Invitrogen Corp.) supplemented with antibiotic mixture (Invitrogen) to achieve final concentration of 100 units of Penicillin/100 μg of Streptomycin/mL.
3. Antennapedia leader peptide CT (Anaspec, Inc.) (ALP-CT) stock solution.
4. Standard 0.65-mL plastic tubes (VWR).
5. Fentanyl/Droperidol.
6. Diazepam.
7. Sodium pentobarbital.
8. 1-mL syringes.
9. Petri dishes.
10. Scissors and forceps.

3. Methods

3.1. Dissection of Lymphatic Vessels from Sprague–Dawley Rats

1. Anesthetize the male Sprague–Dawley rats (weighing between 300 and 400 g) according to anesthesia procedure approved for your AUP (in our studies—by intramuscular injection of the combination of Fentanyl/Droperidol (0.3 mL/kg) and Diazepam (2.5 mg/kg)).
2. Using scissors, make a 4-cm long incision in midline abdomen. Separate fascia and muscle layers. Exteriorize a small loop of intestine, 6–7 cm in length.
3. Take out a section of the mesentery containing lymphatic vessels and then put it into a dissection chamber in DPBS under a dissecting microscope. Identify suitable MLVs and clear all surrounding tissues (see Note 1).

4. Carefully dissect sections of 1–1.5 cm MLVs in length. Then euthanize the rat according to euthanasia procedure approved for your AUP (in our studies—by sodium pentobarbital (120 mg/kg body weight IP)) (see Note 2).
5. To eliminate remains of coagulated blood, rinse the exteriorized vessels in standard 35-mm plastic Petri dishes filled with DPBS under a dissecting microscope.
6. Transfer the vessels to another sterile 35-mm Petri dish filled with prewarmed (38°C) D-MEM/F12 with 100 units/mL Penicillin/100 µg/mL Streptomycin for temporary storage (see Note 3).
7. Lightly touch the edges of segments with forceps on the dish bottom to keep the segment submerged under the solution.

3.2. GFP Reporter Gene Transfection in Isolated Rat Mesenteric Lymphatic Vessels

1. Use recombinant adenoviral constructs (in our studies we used a recombinant adenovirus expressing GFP under the control of the cytomegalovirus immediate-early promoter by the AdEasy system (2), as previously described (3)). Purified virus will be used to transfect lymphatic muscle and/or endothelial cells in whole isolated rat MLV.
2. Apply smallest possible volumes as practical for adenovirus transfection. For this purpose, slightly modify the standard 0.65-mL plastic tubes (Fig. 1a).
3. Separate the caps of the plastic tubes (Fig. 1b) and penetrate several holes in tube.
4. Insert a small diameter plastic tube of ~10 cm length to one hole of plastic cap.
5. Connect the tube with a glass micropipette (~1 cm length) with a tip diameter around 100–120 µm. The other end of the plastic tube will be connected with a 1-mL syringe.
6. Keep three holes in separated tube's body to relieve pressure inside tube after closure, therefore preventing the prolapse of the lymphatic wall inside the micropipette (Fig. 1c).
7. Fill the plastic tube and pipette with D-MEM/F12/a/b and put them into a large plastic Petri dish.
8. Cannulate the lymphatic segments at the upstream end onto the micropipette and secure them at the end of the vessel with 6-0 suture, while the output end remains open.

¹While vessel of interest is still situated in its location in the body, the vessel should be kept at initial natural length and position, so any potential mechanical stress (such as too much distension, wrong direction) during dissection/cleaning procedure will be minimized.

²Cleaning of vessels selected for incision should be done *in situ* as much as possible. Clean the vessel carefully to eliminate as much as possible the adipose tissue and other extracellular matrix elements, which later can attract part of transfections constructs, diminishing therefore the power of transfection agent.

³Procedures must be performed inside biosafety hood to avoid contamination. Everything in contact with the vessel should be single-use sterile (disposable), or alcohol-sterilized (instruments, sutures, vessel chamber, glass pipettes, and glass reservoirs).

9. Mix equal volumes of ALP-CT stock solution to nondiluted adenoviral construct stock solution (1×10^{13} viral particles/mL) for 30 min at room temperature.
10. Dilute this mixture with D-MEM/F12/a/b to produce a final titer of viral particles ranging between 1.1 and 1.5×10^{10} viral particles/mL.
11. Flush the vessel lumen with ~ 0.2 mL of D-MEM/F12/a/b using a 1-mL syringe.
12. For endothelial denudation, if desired—disconnect the syringe from the opened input tube and suck ~ 0.05 mL of air into it. To denude the endothelium, insert air bolus into the vessel lumen. Maintain the vessel for 10–12 s, and then immediately flush it with ~ 0.2 mL D-MEM/F12/a/b with the syringe to completely replace the air with fluid.
13. Inside the lymphatic vessel, replace the D-MEM/F12/a/b solution with ALP-CT/AdGFP-solution using another 1-mL syringe. Tie the vessel at its output end after it is filled with the virus suspension.
14. Carefully fill the $0.65\text{-}\mu\text{L}$ plastic tube with D-MEM/F12/a/b (with or without virus at 1.1×10^{10} viral particles/mL), and then rapidly transfer the vessel, pipette, and plastic cap from the Petri dish into the plastic tube (see Note 4). Under stereomicroscope, make sure that after this procedure vessel is not damaged or bent.
15. Inject the transfection solution containing 1.1×10^{10} viral particles/mL only into nondenuded vessel to transfect only lymphatic endothelial cells.
16. Inject the solution with 1.5×10^{10} viral particles/mL into endothelium-denuded vessels to transfect only lymphatic muscle cells.
17. To transfect both lymphatic endothelial and muscle cells, put the vessels into the solution containing 1.1×10^{10} viral particles/mL, while injecting with the solution containing 1.5×10^{10} viral particles/mL.
18. Put the plastic tube, containing the cannulated vessel, into a tube rack. Open the end of the plastic tube filled with fluid and place it ~ 5 cm above the pipette tip to allow the vessel to be pressurized at all times during the subsequent culture (Fig. 1d).
19. Place the tube rack into a cell culture incubator (10% CO_2 , 37°C) for the required number of hours for transfection.
20. Check the vessels daily. Discard any vessels with visible signs of contamination.
21. Image the vessels with a Confocal-Multiphoton Microscope System to determine the intensity of the signal in cells of the lymphatic wall. The images were acquired at $0.3\text{-}\mu\text{m}$ intervals via 489 nm of peak excitation wavelength and 508 nm peak emission wavelength. Reconstruct the image in different 3D perspectives using the confocal microscope software.

⁴To eliminate the need to recannulate the vessel when air bubbles formed inside the pipettes, tubing or vessel, or later outside vessel while in incubator, the D-MEM/F12/a/b solution should be continuously heated during all vessel setup procedures to 38°C .

Acknowledgments

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Fig. 1.

Preparation of simple 0.65- μ L plastic tube-based setup for gene transfection of single vessel. (a) Intact plastic 0.65- μ L plastic tube. (b) Plastic cap and tube separated. (c) Holes were penetrated: one hole in plastic cap allowed insertion of a small diameter plastic tubing ~10 cm length. This tubing from the inner cap side was connected with ~1 cm length glass micropipette with a tip diameter of ~100–120 μ m. Three holes in separated tube's body helped to relieve pressure inside tube after closure, therefore preventing the prolapse of the lymphatic wall inside the micropipette. Surgical 12-cm scissors presented to reflect dimensions. (d) Assembled tube with isolated rat MLV loaded for gene transfection experiment set in tube rack ready for incubation. Other end of tubing (upper) finally opened to atmosphere.