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Study of the endogenous CRAC channel using shRNA-mediated gene silencing

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Summary

The Ca^{2+} release-activated Ca^{2+} (CRAC) current is a major signaling event in non-excitabile cells whereby Ca^{2+} store depletion activates Ca^{2+} entry across the plasma membrane from the extracellular space. Stromal interaction molecule 1 (STIM1) and Orai1 proteins are the key molecular players of the CRAC channel. Previous studies have linked activity of this channel to many physiological functions and dysregulation of the CRAC channel has been associated with various diseases. In the absence of inducible tissue-specific knockout mice, *in vivo* knockdown studies examining the endogenous function of CRAC channel proteins, STIM1 and Orai1 is a challenge. In this chapter, we describe a lentiviral-delivery system of shRNA-mediated gene silencing that has proven successful in studying the endogenous CRAC channel *in vivo*.

Keywords

CRAC; STIM1; Orai1; ShRNA; Gene silencing; Lentiviral particle; Western blot

1. Introduction

STIM1 is Ca^{2+} sensor in the endoplasmic reticulum (ER) membrane that senses Ca^{2+} depletion of ER Ca^{2+} stores, [1, 2] while Orai1 residing in the plasma membrane is the pore-forming subunit of CRAC channels [3–5]. Both STIM1 and Orai1 are necessary for activation of CRAC channels. Recently, CRAC channel activity has been shown to regulate various physiological functions and dysregulation of CRAC channel activity has been linked to a number of diseases [6–11]. Identifying how aberrant CRAC channel activity causes human disorders requires *in vivo* knockdown studies of either STIM1 or Orai1 proteins. Due to the practicality of certain studies in bigger species other than mice (*e.g.* rats) and due to the limitation of siRNA and electroporation techniques *in vivo*, we have used a method of lentiviral-delivery of short hairpin RNA to knock down STIM1 or Orai1 protein expression in rat arteries. Here, we describe a detailed procedure of how to produce lentivirus-expressing shRNA, and validate the efficiency of protein knockdown using western blotting. We also confirmed delivery efficiency by carrying out a functional assay using low noise

whole-cell patch clamp recordings (procedure not shown) [12, 13] to characterize the attenuation of CRAC currents in infected cells.

2. Materials

2.1 Materials and Solutions for Producing Lentiviral Vectors

1. 293FT cells (Invitrogen)
2. Plasmid DNAs: pCMV-VSVG envelope plasmid, pCMV-dR8.2 2nd generation lentiviral packaging plasmid, pFUGW-GFP-shSTIM1/pFUGW-GFP-shOrai1/pFUGW-GFP-shLuciferase lentiviral plasmids (shLuciferase used as control shRNA). (*see* Note 1)
3. PolyJet transfection reagent (SignaGen)
4. Opti-MEM I reduced serum medium (Opti-MEM)
5. DMEM medium
6. Fetal bovine serum (FBS)
7. Penicillin/Streptomycin
8. L-Glutamine
9. Poly-D-lysine (0.1 mg/mL solution of Poly-D-lysine hydrobromide mol wt 30,000–70,000)
10. 100 mm cell culture dishes
11. 15 mL centrifuge tubes
12. 50 mL centrifuge tubes
13. Amicon ultra 15 mL filters (Amicon Ultra-15, membrane Ultracel-PL, 30 kDa)
14. Complete medium: 445 mL DMEM, 5 mL 100x L-Glutamine, 50 mL FBS
15. 2% serum medium: 485 mL DMEM, 5 mL 100x L-Glutamine, 10 mL FBS

2.2 Materials and Solutions for Western Blotting

1. Nonfat dry milk
2. Fiber pads
3. Polyvinylidene difluoride membrane
4. BCA protein assay kit (Pierce)
5. PAGE electrophoresis apparatus
6. Mini Trans-Blot Electrophoretic Transfer Cell⁷.
7. Protease inhibitor cocktail

¹pCMV-VSVG, pCMV-dR8.2 and pFUGW-GFP are available at addgene. For knockdown of proteins STIM1, Orai1 and non-targeting control, three individual transfections (of 10×100mm dish each) are necessary.

8. Phosphatase inhibitor cocktail
9. PBS solution: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4.
10. RIPA lysis buffer: 150 mM NaCl, 1% Triton X-100, 0.2 mM EDTA, 0.1% SDS, 0.5% Sodium deoxycholate, 50 mM Tris-HCl, pH 8.0.
11. 5x SDS loading buffer: 250 mM Tris-HCl, pH 6.8, 10% SDS, 30% Glycerol, 5% β -mercaptoethanol (or 0.5 M DTT), 0.25% bromophenol blue.
12. 10% polyacrylamide gel (9.6 mL): 3.2 mL 30% acrylamide, 4 mL H₂O, 2.4 mL 1.5 M Tris-HCl, 48 μ L 20% SDS, 32 μ L 10% APS, 4.8 μ L TEMED. (*see Note 2*)
13. SDS-PAGE running buffer: 25 mM Tris-HCl, 192 mM glycine, 0.1% SDS, pH 8.3.
14. Transfer buffer: 20% methanol, 25 mM Tris, 192 mM glycine, pH 8.3.
15. Tris-buffered saline (TBS): 150 mM NaCl, 10 mM Tris-HCl, pH 8.0.
16. TBST: TBS with 0.1% Tween 20.
17. Blocking buffer: 5% nonfat milk in TBST.

3. Methods

3.1 Lentiviral Vector Production

Day 1 – Seed 293FT cells (*see Note 3*)

1. The day before transfection, seed 293FT cells into 10× 100 mm cell culture dishes (*see Note 4*) at a density of 4×10^6 cells per dish supplying 6 mL complete medium (*see Note 5*).
2. Incubate the cells for the next 24 h in a 37 °C, 5% CO₂ tissue culture incubator.

Day 2 – Transfection

1. Check and wait until cells reach 80–90% confluence to do transfection.
2. Warm Opti-MEM medium and complete medium in a 37 °C water bath. Thaw plasmids, pCMV-VSVG, pCMV-dR8.2, pFUGW-GFP-shSTIM1, pFUGW-GFP-shOrai1, pFUGW-GFP-shLuciferase on ice.
3. Change the medium of each 100 mm dish with 5 mL of fresh complete medium (30–60 min before transfection).

².Acrylamide is considered a potential occupational carcinogen by U.S. government agencies so, carefully prepare the gel.

³.The 293FT cell line is a fast-growing, highly transfectable clonal isolate derived from human embryonal kidney cells transformed with the SV40 large T antigen. When a ViraPower™ expression vector and the ViraPower™ Lentiviral Packaging Mix are co-transfected into 293FT cells, high levels of the viral RNA and the gag/pol and rev proteins required for packaging are produced.

⁴.In order to enhance adherence to the substrate, pre-coat the 100mm cell culture dishes with Poly-D-lysine. (Simply add 10 mL 1x Poly-D-lysine solution into each dish. After 20 min room temperature incubation, remove the liquid by vacuum.)

⁵.Make sure that each dish contains the same number of cells and that the cells are evenly distributed throughout the bottom of the dishes.

4. Prepare transfection complexes solution for 10× 100 mm dishes (*see Note 1*) Mix 1: 20 µg pCMV-VSVG, 40 µg pCMV-dR8.2, 40 µg p-shRNA and 5 mL Opti-MEM medium in a 15 mL tube [2]. Mix gently by swirling the pipet tip around or tapping with a finger.
5. Mix 2: 30 µL PolyJet transfection reagent and 5 mL Opti-MEM medium in another 15 mL tube [1]. Mix gently by swirling the pipet tip around or tapping with a finger.
6. Add the diluted PolyJet transfection reagent (tube #2) immediately to the diluted plasmid DNA (tube #1) all at once (*see Note 6*). Mix gently. Incubate the solution on standing for ~15 min at room temperature.
7. Add the transfection complexes (1 mL) dropwise onto the medium in each dish and homogenize the mixture by gently swirling the plate (*see Note 7*). Incubate the cells at 5% CO₂, 37 °C.
8. Replace the transfection medium by fresh 2% serum medium (6 mL/dish) 6–8 h post transfection.

Day 3 – Observe the transfected cells

1. Observe the cells under a fluorescence microscope. Transfection efficiency can be assessed visually by GFP marker. Ideally, transfection efficiency should be >90%.

Day 4 – First virus harvest

1. Sterilize the Amicon filters by UV light (*e.g.* using the UV-light of a cell culture hood) for at least 30 min.
2. Collect the medium from 10 dishes with 50 mL tube(s). Centrifuge at 500 g for 5 min at room temperature to remove cell debris. Store the cleared medium at 4 °C.
3. Add 6 mL of fresh 2% serum medium to each dish. Incubate the cells at 5% CO₂, 37 °C for another 24 h.

Day 5 – Second virus harvest

1. Collect the medium from the 10 dishes again. Centrifuge at 500 g for 5 min at room temperature to remove cell debris, then put the first harvest and the second harvest together and store at 4 °C. (Usually, at least 2× 50 mL viral supernatant are able to be obtained from two single day.)

Day 6 – Concentrate virus

1. Add 15 mL viral supernatant to Amicon ultra 15 mL filter.

⁶Do not mix the solutions in the reverse order.

⁷It is better to finish the transfection step at early morning or middle night, so operator is flexible to remove the transfection medium 6–8 h post transfection.

2. Cap the filter loosely and place it into a swing bucket centrifuge. Centrifuge tubes at 1000 g for 30 min at room temperature. Repeat step 15 and 16 with the same filter until all of the 50 mL viral supernatant has been concentrated. The ideal final volume of concentrated virus should be approximately 250 μ L for each 50 mL viral supernatant.
3. Aliquot concentrated virus and store at -80°C .

3.2 Lentiviral particles validation

Before conducting *in vivo* experiments, cultured vascular smooth muscle cells are infected by either shOrai1, shSTIM1 or shLuciferase lentiviral particles (*see* Note 2).

Two days before transfection, seed vascular smooth cells into 6 well plates at a density of 0.1×10^6 / well. Add 10 μ L lentiviral particles into each well, and shake well to make sure most cells could be infected by virus (*see* Note 8).

Five days later, over 90% of cells were expressing GFP as evidenced by green fluorescence (*see* Note 9). We documented protein knockdown on cultured vascular smooth muscle cells using western blot (*see* Figure 1A).

3.3 Lentiviral particles infection In vivo

Validated lentiviral preparations are then used to perform *in vivo* knockdown of Orai1 (and/or STIM1) proteins in medial vascular smooth muscle cells from carotid arteries subjected to a balloon angioplasty protocol. The balloon injury protocol is widely accepted for the study of vascular smooth muscle proliferation and migration *in vivo*. We used this procedure to determine whether the prevention of the up-regulation of Orai1 (that occurs in response to vascular injury) could prevent vascular smooth muscle proliferation and migration, and subsequent neointimal formation. During vascular balloon injury surgery, 50 μ L solution of lentiviral particles encoding either shOrai1 or shLuciferase are infused into the injured segment of the common carotid artery and incubated for 30 min (*see* Note 10). After incubation, the final steps of balloon injury surgery are performed. A detailed procedure of balloon injury surgery, may be viewed in a video publication from our lab [14]. After two weeks, the injured tissue is isolated to perform either western blot or H&E staining assays. Below is our western blotting procedure.

3.4 validation of In vivo CRAC channel knock-down by Western blotting

1. For injured vessels, clean carotid arteries with ice-cold PBS solution to remove all blood clots from the lumen. For intact control vessels, carefully remove the endothelial layer. Separate the medial and neointimal layers from the adventitial layer by carefully peeling off adventitia. Then Subject tissues to protein extraction or wrap them up with a small piece of aluminum foil to store in liquid nitrogen storage tank for future use.

⁸.A similar procedure can be applied on other types of cells.

⁹.Take pictures on the infected cells with fluorescence microscope to define transfection efficiency.

¹⁰.Leftover viral solutions in lumen have to be removed to avoid dissemination of viral solutions into the systemic circulation.

2. Cut into small pieces the harvested vessel and ground them in a Teflon homogenizer with 150 μ L of RIPA lysis buffer on ice. (*see* Note 11 and 12)
3. Sonicate (to use Homogenizer with 30% power, few 10 seconds pulse until liquid becomes clear) and centrifuge (20,000 g, 4 °C for 5 min) the samples then determine protein concentration using the BCA protein assay kit (Pierce).
4. Load the protein samples in a 10% polyacrylamide gel (10 μ g/lane), and run the gel at 180 mV.
5. After electrophoresis, transfer proteins onto a methanol-pre-activated polyvinylidene difluoride membrane using electrophoretic transfer at 50 mV for 1 hour 45 min.
6. Block the membrane with blocking buffer for 4–6 h at room temperature with shaking.
7. After blocking; without washing, place the membrane in blocking buffer with the anti-Orai1 antibody dilution and incubate “overnight” at 4 °C in a shaker. (*see* Note 13)
8. After primary antibody incubation, rinse the membrane twice with TBST, and wash it 3 times with TBST for 3 min.
9. Incubate the membrane for 2 h in a blocking buffer with HRP-conjugated secondary antibody (diluted according to the manufacturer’s protocol).
10. After the secondary incubation, rinse the membrane twice with TBST, and wash it 3 times with TBST for 5 min.
11. Before developing the blot, rinse the membrane 3 times with TBST buffer. The membrane is then developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).
12. Typical results show that Orai1 protein expression is significantly down regulated in vessels infected with viral particles encoding shOrai1, whereas infection with shluciferase do not affect Orai1 protein expression (*see* Figure 1B). (*see* Note 14)

11. Add 10% protease inhibitor cocktail and 10% phosphatase inhibitor cocktail to RIPA lysis buffer immediately before use.

12. Pick lysing buffer properly, please see below:

Protein location	Buffer recommended
Membrane bound	RIPA or NP-40
Whole cell	RIPA or NP-40
Cytoplasmic	Tris-HCl
Nuclear	RIPA or use nuclear fraction protocol
Mitochondria	RIPA or use mitochondrial fraction protocol

13. We use anti-Orai1 antibody from (Alomone) at 1:2000 but note that the optimal dilution for a specific antibody will vary and should be determined by the end-user. See the product-specific specification sheet for recommended starting dilutions.

14. *Functional confirmation of the efficiency of gene silencing and CRAC current inhibition by patch clamp recording.* We have performed whole cell patch clamp recording on the cells isolated from either neointima or media of the injured area of artery. The results showed evidence that the current we measured is CRAC current due to the fact that: i) Store depletion induced by BAPTA activated an inward Ca^{2+} current; ii) the current can be amplified by DVF bath solution; iii) Under DVF bath solution, the current

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showed unique depotentiation. The results also showed that Orai1 knockdown completely inhibited the CRAC current, while non-targeting control didn't show any effects (see Figure 2).

Figure 1.

A. Western blot analysis of cultured vascular smooth muscle cells infected for 5 days in culture with either ShOrai1 or shLuciferase control lentiviruses; shOrai1 reduces Orai1 expression **B.** Western blot analysis from injured and non-injured vessels show that Orai1 expression is upregulated 2 weeks after vascular injury. ShOrai1 lentiviruses caused efficient Orai1 protein knockdown compared to shLuciferase control 2 weeks after injury and lentiviral transduction.

Figure 2.

Whole-cell patch clamp recordings from vascular smooth muscle cells isolated from the media of injured vessels infected with shOrai1 or shLuciferase lentiviruses show that shOrai1 lentiviruses abrogated CRAC current activation compared to shLuciferase control. Figure B and D are I-V curves taken from traces in A and C where indicated by asterisks, respectively. Note that patch clamp recordings are performed on cells that are positively infected as evidenced by the GFP fluorescence.