Chapter 5

Small Interfering RNA-Mediated Connexin Gene Knockdown in Vascular Endothelial and Smooth Muscle Cells

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Abstract

Global knockout of vascular connexins can result in premature/neonatal death, severe developmental complications, or compensatory up-regulation of different connexin isoforms. Thus, specific connexin gene knockdown using RNAi-mediated technologies is a technique that allows investigators to efficiently monitor silencing effects of single or multiple connexin gene products. The present chapter describes the transient knockdown of connexins in vitro and ex vivo for cells of the blood vessel wall. In detail, different transfection methods for primary endothelial cells and ex vivo thoracodorsal arteries are described. Essential controls for validating transfection efficiency as well as targeted gene knockdown are explained. These protocols provide researchers with the ability to modify connexin gene expression levels in a multitude of experimental setups.

Key words Transfection, Lipofection, Electroporation, siRNA, Off-target, Knockdown, Ex vivo transfection

1 Introduction

RNA interference (RNAi) is a naturally occurring mechanism that was first described in *Caenorhabditis elegans* [1, 2]. Since then, studies about the mechanisms and molecular machinery have been carried out in a number of model organisms [3, 4]. There are two main RNAi pathways that differ in the origin of interfering RNA molecules and the mechanisms of action; however, both mechanisms produce similar outcomes. The basic underlying mechanism is derived from a host defense mechanism that exists to prevent foreign RNA viruses from replicating inside the host cells [5]. Host cells can recognize the viral double-stranded RNA (dsRNA) by Dicer, a cytoplasmic ribonuclease, which cuts dsRNA into small interfering RNAs (siRNAs) of 21–25 bp in length to prevent the

Mathieu Vinken and Scott R. Johnstone (eds.), *Gap Junction Protocols*, Methods in Molecular Biology, vol. 1437,
DOI 10.1007/978-1-4939-3664-9_5, © Springer Science+Business Media New York 2016

translation of viral RNA into protein [6–8]. Another mechanism of RNA interference is the internal regulation of gene expression by another class of siRNAs, the micro RNAs (miRNAs) with 19–25 bp in length. miRNAs are encoded in the genome, synthesized in a multistep process, and cleaved into active forms of RNA by Dicer [9, 10]. Both types of interfering RNAs (i.e., siRNA and miRNA) induce the formation of the RNA-induced silencing complex (RISC) in the cytoplasm. The RISC complex binds the double-stranded siRNA/miRNA, unwinds the RNA, and binds the guide strand, which is complementary to the targeted mRNA. In a subsequent step, the RISC complex binds targeted mRNA, which leads to the degradation, cleavage, or blocking of mRNA preventing the translation of mRNA to protein [11–13]. One main difference between the two different interfering RNAs is that siRNAs have complete sequence specificity compared to miRNAs and results in the physical cleavage of the siRNA-targeted mRNA [14–16].

To induce targeted knockdown of a gene of interest (GOI), researchers can use the RNAi mechanism by exogenously applying interfering RNAs to induce the sequence-specific blockade or degradation of mRNA with subsequent translational silencing [17, 18]. In general, there are three different ways to induce RNAi: (1) commercially available double-stranded siRNAs designed with perfect complementarity and unique sequences to the targeted mRNA that have to be exogenously applied and introduced into the cell; (2) plasmids encoding short hairpin RNAs (shRNAs), which are the preprocessed form of siRNAs, can also be used to induce the RNAi effect; or (3) viral-based vectors that express shRNAs can be introduced into difficult to transfect cells or tissues and can provide long-term gene silencing [1, 19, 20]. These given RNAi-inducing systems must be carefully selected to match the appropriate silencing method with the experimental specimen type.

Ultimately, the success of RNAi experiments depends on the specificity and efficiency of the siRNA. Different companies such as GE Healthcare Dharmacon, Inc. or Ambion Thermo Fisher Scientific® have developed specific design algorithms to identify potent siRNA sequences to offer a library of well-validated predesigned siRNAs. However, highly complementarity siRNA sequences can still induce off-target effects [21, 22] (*see* **Note 1**). Pooling of several independent siRNAs that target the same gene is a common strategy that has been shown to yield efficient gene silencing while reducing the frequency of off-target effects [23]. Furthermore, transfection efficiency and cell survival is another important step for a successful RNAi gene expression regulation.

Cell lines, primary cells, and tissue differ in their ability to be transfected. Here we present a classical transfection protocol for primary endothelial cells, which can be adjusted to a number of different cell types. We also present a modified siRNA-mediated

gene silencing protocol for ex vivo endothelial and smooth muscle targeted gene knockdown of intact blood vessels [24]. These two different methods allow the researcher to adjust the experimental setup to the investigated cell type, cell line, or tissue and to efficiently silence their GOI. Although outside the scope of these protocols, it should be noted that deep-tissue applications or long-term RNAi-based gene silencing strategies should be adjusted to the experimental setup by the use of laser- or viral-based transfection systems [25–28].

Often multiple isoforms from a family of genes are expressed within the same cell resulting in difficulty in isolating the unique contributions of each protein to the function of the cell. Such is the case with connexins, which also have been shown to change their expression patterns in isolated primary cells. Knockdown of a specific or multiple connexin(s) allows for isolated functional analysis for each isoform [29, 30]. Thus, the current chapter describes a mechanism for knocking down GOIs in both primary cells and ex vivo blood vessels.

2 Materials

2.1 Cell Culture of Primary Vascular Endothelial Cells

1. Human umbilical endothelial cells (HUVEC) purchased at passage 2 (*see* **Note 2**).
2. Endothelial cell medium: Medium 200 supplemented with low serum growth kit (Thermo Fisher Scientific) and 20% FBS (*see* **Note 3**).
3. Incubator at 37 °C, 5% CO_2, 90% humidity, and class 2 biosafety cabinet.
4. Sterile cell culture 6- or 12-well plastic plates.
5. 50 μM fibronectin solution (*see* **Note 4**).
6. Plastic cell scraper.
7. Trypsin–EDTA.
8. Sterile PBS: 137 mM NaCl, 2.7 mM KCl, 1.5 μM KH_2PO_4, 8.1 mM Na_2HPO_4 without divalent cations, e.g., $CaCl_2$ or $MgCl_2$.
9. Light microscope with 10× objective.

2.2 Transfection of Endothelial Cells

1. Lipofectamine® 3000 (*see* **Note 5**).
2. Opti-MEM media.
3. Transfection media: Medium 200 supplemented with low serum growth kit (Thermo-Fisher Scientific) and 0.1% FBS (*see* **Note 6**).
4. siRNAs (*see* **Note 7**).

2.3 Dissection and Transfection of Mouse Thoracodorsal Arteries (TDAs)

1. Model organism: mouse.
2. Dissecting microscope with 40× zoom capability.
3. Sterile microdisscetion tools (pair of fine forceps, dissection scissors, etc.)
4. Krebs-HEPES physiological salt solution: 118.4 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 4 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 10 mM HEPES, 6 mM Glucose, 2 mM $CaCl_2$ pH to 7.4 and use with or without 1% Bovine Serum Albumin Fraction V (*see* **Note 8**).
5. Culture myograph 202CM vessel cannulation system (Danish MyoTechnologies) or other vessel cannulation system including glass micropipettes for vessel cannulation.
6. 10-0 Nylon sutures.
7. siRNAs (*see* **Note 7**).
8. Nucleofector™ Cell Line Kit for Human Coronary Artery Endothelial Cells (HCAECs) and Human Aortic Smooth Muscle Cells (hAoSMCs) (Lonza, Swiss).
9. Nucleofector™2b Device (Lonza, Swiss) for cell electroporation or similar device and corresponding electroporation cuvettes.
10. RPMI medium 1640 with or without 1% Bovine Serum Albumin Fraction V, 1% L-glutamine, 1% penicillin/streptomycin (*see* **Note 8**).
11. Incubator at 37 °C, 5% CO_2, 90% humidity, and a class 2 biosafety cabinet (cell culture hood).
12. 100 mm sterile cell culture plastic dishes or 24-well plates.

3 Methods

3.1 Setting Up Plates of Vascular Cells for Experiments

1. For HUVECs only, place 12.5 μL or 25 μL of fibronectin in the middle of each well of a 12- or 6-well plate, respectively, and use a cell scraper to spread the solution around the surface of the well.
2. Leave the plate in the cell culture hood to allow the fibronectin to dry.
3. Once dry, plates can be used immediately or sealed in plastic bags and stored at 4 °C for 1–2 weeks for later use.
4. At 80–90% confluence, remove cell media from flasks of cells and wash cells two times with 5 mL of PBS.
5. Pipette in 1× Trypsin–EDTA. Rock the flask to cover the cells and place back in the 37 °C incubator for 3 min.
6. Check for cell dissociation by microscope.

7. Once 80–90% of cells have become dissociated add back at least twice the amount of media than Trypsin–EDTA to cells. For continued passage add one-fifth of cells back to the flask and top up with fresh media.
8. Count the remaining cells using an hemocytometer and adjust the volume to approximately 2×10^4 cells per mL. This will ensure confluence in 4–5 days (*see* **Note 9**).
9. Place 0.75 or 1.5 mL of cells in each well of a 12- or 6-well plate, respectively, and place plate in 37 °C incubator.
10. Incubate overnight.

3.2 Transfection of HUVEC Cells

1. Remove siRNA from the −80 °C and place on ice to thaw.
2. Prepare 1.5 mL microcentrifuge tubes for siRNA dilution, siRNA, and Lipofectamine additions.
3. Dilute 50 μM stock siRNA 1:10 in Opti-MEM to a concentration of 5 μM (*see* **Note 10**).
4. Prepare siRNA tubes and Lipofectamine tubes by adding 50 μL of room temperature Opti-MEM to each tube per experiment.
5. For 6-well plates (per well), pipette 1 μL of diluted stock siRNA into siRNA tube containing 50 μL Opti-MEM. For 12-well plates (per well), pipette 0.5 μL of diluted stock siRNA into siRNA tube containing 50 μL Opti-MEM.
6. For 6-well plates (per well), pipette 1.0 μL Lipofectamine into the Lipofectamine tube containing 50 μL Opti-MEM. For 12-well plates (per well), pipette 0.5 μL Lipofectamine into the Lipofectamine tube containing 50 μL Opti-MEM.
7. Add the Lipofectamine mix to the siRNA mix and swirl with the pipette tip to mix (*see* **Note 11**).
8. Allow mixed solution to stand for 5–10 min.
9. Remove media from cells and replace with transfection media. For 6-well plates, add 1.9 mL transfection media to each well. For 12-well plates, add 0.9 mL transfection media to each well.
10. Add 100 μL of the siRNA: Lipofectamine mix to each well for a final volume of 2 mL (for 6-well plates) or 1 mL (for 12-well plates) and for a final siRNA concentration of 2.5 nM.
11. Place plate back into the 37 °C incubator for 24 h.
12. After 24 h, remove transfection media and replace with endothelial cell media.
13. Incubate for a further 24 h (*see* **Note 12**).
14. Analyze cell viability and transfection efficiency before using cells in experimental preparations (*see* **Note 13**).

3.3 Dissection of Mouse TDA

1. Euthanize mice (8–10 weeks old) using CO_2 asphyxia and place in the lateral decubitus position at a clean surgical station with dissecting microscope.
2. Spray scapular area with 70% ethanol to minimize fur getting into the exposed tissue.
3. Make a 3–4 cm long incision on one side in the scapular region using forceps and a dissecting scissor [31].
4. Carefully resect the section of skin covering the latissimus dorsi muscle. Be careful not to disrupt the superficial dorsal muscle underneath the latissimus dorsi (*see* **Note 14**).
5. Remove the latissimus dorsi by microdissection to expose both the superficial dorsal muscle and TDA, which feeds the spinotrapezius muscle. The TDA is surrounded on both sides by veins (*see* **Note 15**).
6. Isolate the TDA (~10–15 mm) by carefully dissecting away surrounding fat and veins. Place TDA in 1.5 mL microcentrifuge tube containing cold Krebs-HEPES on ice. TDA can remain on ice in cold Krebs-HEPES for up to 6 h.
7. Repeat **steps 2–5** for isolating contralateral TDA.

3.4 Ex Vivo Endothelial or Smooth Muscle Cells Transfection

1. Transfer a freshly isolated TDA to the prefilled bath chamber of a pressure myograph (Danish MyoTechnology) with room temperature Krebs-HEPES solution without flow. Make sure pressure myograph tubing and glass micropipettes are prefilled with Krebs-HEPES supplemented with 1% BSA solution and verify that the tubes and micropipettes do not have any air bubbles (*see* **Notes 8** and **16**).
2. Gently open each end of the TDA with fine forceps and pull each TDA end on opposing glass cannula ends within the chamber. Only handle vessel ends to minimize mechanical stress and float the vessel onto the micropipettes.
3. Secure artery-cannula with 10-0 nylon suture (*see* **Note 17**).
4. Attach the myograph tubing to the pressure transducer and perfuse vessel lumen with Krebs-HEPES 1% BSA solution for 2 min by turning on the pressure transducer and increasing the inflow pressure to 40 mmHg and set the outflow pressure to 0 mmHg. This will clear residual red blood cells and debris.
5. Mix 82 μL Nucleofection reagent for HCAEC with 18 μL kit supplement 4 and dilute siRNA reagents to final concentration (100 nM or 10 pmol siRNA/sample) in a 1.5 mL microcentrifuge tube (*see* **Note 18**). If only transfecting smooth muscle cells, and not the endothelial cells, use the nucleofection solution for hAoSMC and do not include any siRNA (*see* **Note 19**).
6. Incubate nucleofection solution/siRNA mix for 10 min at room temperature.

7. Disconnect the inflow tubing from the pressure transducer box first and then disconnect that tube from the glass micropipette. This must be performed specifically in this sequence to stop flow through the TDA.
8. Using a 1 mL syringe, completely fill the disconnected inflow tubing with the HCAEC nucleofection solution/siRNA mix. Add a small air bubble to the inflow tube on the side that will be attached to the pressure transducer.
9. Reconnect the inflow tubing to the pressure transducer box and then to the micropipette, specifically in that sequence. Avoid introducing air bubbles directly into the glass micropipette (*see* **Note 20**).
10. Perfuse the vessel lumen with HCAEC nucleofection solution/siRNA by increasing the inflow pressure to 40 mmHg and leaving outflow pressure at 0 mmHg. The bubble near the pressure transducer should move toward the glass micropipette. Allow for the new solution to move through the vessel (the bubble should move at least the length of the glass micropipette to verify the nucleofection solution/siRNA has moved into the vessel (*see* **Note 21**).
11. Quickly and simultaneously remove the vessel from the inflow glass micropipette and tie off the vessel ends with the anchoring suture. Repeat for the opposite end to trap reagents inside vessel lumen.
12. If transfecting only endothelial cells, fill an electroporation cuvette provided with the Nucleofection kit with 100 μL of HCAEC Nucleofection reagent without siRNA (82 μL Nucleofection Reagent for HCAEC with 18 μL kit supplement 4). If transfecting smooth muscle cells, fill the provided electroporation cuvette with hAoSMC Nucleofection reagent (84 μL HAoSMC Nucleofection reagent supplemented with 18 μL of supplement) with siRNA diluted to final concentration. Let this incubate in the cuvette for 10 min at room temperature.
13. Gently transfer the ligated artery to the electroporation cuvette (*see* **Note 22**).
14. Electroporate vessel using program A-034 on Nucleofector 2b device. New protocols for the Nucleofector 4D line are available through Lonza (*see* **Note 23**).
15. Let vessel recover for exactly 5 min in cuvette (*see* **Note 24**).
16. Remove vessel from electroporation cuvette and place in myograph bath filled with Krebs-HEPES solution and carefully remove old sutures with fine forceps. Position vessel ends on opposite micropipettes using new 10-0 nylon sutures. Minimize vessel handling.

17. Flush vessel lumen with Krebes-HEPES 1% BSA solution by increasing the inflow pressure to 40 mmHg for 1 min.
18. Perfuse vessel lumen with warm (37 °C) RPMI 1640 media supplemented with 1% BSA, 1% L-glutamine, and 1% penicillin/streptomycin using an inflow pressure of 40 mmHg by adding this media to the inflow tubing as previously described.
19. Remove vessel from micropipettes (without ligating ends; no sutures) and place in a sterile culture dish or 24-well plate containing RPMI 1640 media supplemented with 1% BSA, 1% L-glutamine, and 1% penicillin/streptomycin (prewarmed to 37 °C) for 18–24 h at 37 °C and 5% CO_2 in humidified cell culture incubator (*see* **Note 25**).
20. Analyze cell viability and transfection efficiency before using vessels in experimental preparations (*see* **Note 13**).

4 Notes

1. To test for off-target effects, nontargeted siRNA are generally used, which consist of a scrambled sequence of the siRNA such that the scrambled sequence doesn't target the target mRNA. This negative control is used to show that the actual procedure including the transfection, introduction of siRNA into the cell, and activation of cellular machinery for RNAi does not affect the mRNA expression or functional output being tested.
2. Primary HUVEC cells are supplied in frozen vials from the manufacturer and should be stored at −80 °C until use.
3. The low serum growth kit from Thermo-Scientific contains 1 mL gentamycin/amphotericin B (500×), a 1 mL mixture of basic fibroblast growth factor (1.5 μg/mL)/heparin (5 mg/mL)/BSA (100 μg/mL), 0.5 mL hydrocortisone (1 mg/mL), 1 mL EGF (5 μg/mL), and 5 mL of FBS. To make the endothelial cell media, we add all of the low serum growth kit with the exception of the FBS (which is not used) to the 500 mL of M200 media, mix for 10 min at room temperature, remove 100 mL (*see* **Note 6**), and replace with 100 mL of FBS that has been batch tested for cell growth.
4. Fibronectin coating of plates promotes adhesion and reduces the tendency of the endothelial cells to float off from the plate surface.
5. Lipofectamine 3000 was selected due to good transfection efficiency as compared to Lipofectamine 2000 in these cells. Other transfection reagents and techniques, e.g., nucleofection may also produce good results in these cells.

6. The 100 mL of no-serum media (removed while making up the stock endothelial cell media) has 50 μL of FBS added to make a final concentration of 0.1% serum, which is useful for cell transfections and drug treatments and can sustain HUVEC for several days in culture without cell death.
7. Desiccates of siRNA (5 nM) from company should be mixed with 100 μL of DNase free water to a final concentration of 50 μM. siRNAs can then be stored at −80 °C and freeze-thawed up to 50 times without loss.
8. BSA supplemented buffers and media should be sterile filtrated, if they are used in combination with glass micropipettes. Otherwise the BSA will clog the micropipettes.
9. Optimally, cells should never exceed 95% confluence. Experiments described here require a longer time course, e.g., 3–4 days and should be seeded to reach around 95% confluence at the end of the experiment. If cells become too confluent, they can detach which will affect the results of the experiments.
10. Recommended usage is 2 nM (1:25,000) to 10 nM (1:5000) from the original 50 μM stock so it is best to dilute the main stock first.
11. Gentle mixing should be used to avoid pipetting up and down.
12. Optimal knockdown occurs between 48 and 72 h after transfection depending on the half-life of the protein of interest (*see* **Note 13**).
13. Cell viability after transfection should be tested and can be assessed by using different commercially available kits. The lactate-dehydrogenase (LDH) Cytotoxicity Assay measures the release of the cytoplasmic enzyme, LDH, into the extracellular space/vessel lumen, which is an indicator for cell death. Trypan blue 0.4% assay can be used after transfection to monitor the viability of cells. This dye is membrane impermeable to healthy cells but can diffuse across the plasma membrane in dying cells. Additionally, transfection efficiency should be determined. By transfecting fluorophore-coupled non-target siRNAs, the amount of fluorescent cells can be easily determined with a fluorescent microscope. The transfection of a GFP-expressing plasmid can also be used as a control for transfection efficiency, but because of the difference in size, there is no direct comparison possible to the actual amount of siRNA-transfected cells. The successful knockdown of the gene of interest should be determined in at least two ways: on the level of mRNA and protein, for example, via PCR and Western Blot, respectively. The degradation of mRNA could be tested within 24–48 h. It should be taken into account that some ways of gene silencing do not cleave the mRNA and, thus, intact

mRNA could be detected although the translation into protein is inhibited. The detection of the protein of interest via Western Blot is an alternative to test for the mRNA silencing. Depending of the half-life of the investigated protein, protein should be isolated between 48 and 72 h. Connexins with a half-life of 4–5 h could show an effect after 24 h. The appropriate time of knockdown has to be determined for each protein and cell type.

14. Remove enough skin and expose a decent size working area. Keep tissue hydrated by flooding the area with cold Krebs-HEPES supplemented with 1% BSA solution during dissection.
15. TDA is the center vessel of the three parallel blood vessels lying on top of the superficial dorsal muscle.
16. Glass micropipettes should have a diameter around the same size of the TDA vessel or slightly smaller (around 240 μm).
17. Sutures should be preknotted with a single overhand knot and loosely wrapped over the micropipettes before placement of the vessel; two sutures per side are optimal to prevent the vessel from being pulled off of the micropipettes. Tie the sutures over the vessel on the first side that is cannulated and then slide the second side of the vessel onto the other micropipette and tighten the suture over the second side of the vessel.
18. Recommended usage is between 50 and 300 nM (5–30 pmol siRNA/sample).
19. Use HAoSMC kit for smooth muscle cell transfection. In this case, only HAoSMC Nucleofection reagent (with no siRNA) is perfused and trapped in the vessel lumen. For smooth muscle cell transfection, siRNA constructs are mixed in the electroporation cuvette before the vessel is placed inside.
20. When reconnecting the inflow tubing to the glass micropipette, slightly overfill the tube with transfection solution so that a droplet remains on the tip of the tube to be connected to the glass micropipette. Reconnect the inflow tube such that there is no air bubble added to the glass micropipette.
21. Visually observe the filling of the vessel with transfection reagent/siRNA. Only a small amount is necessary to completely fill the vessel.
22. Make sure that the ligated vessel is completely covered with solution.
23. The HCAEC Nucleofection kit is supported by the Nucleofector 2b device and is used for endothelial cell transfection. New reagents and protocols exist for the Nucleofector 4D line of electroporation devices (Lonza) [32].

24. Following transfection, the viability of the vessel is improved when the cells can briefly recover without disturbances for 5 min. However, after 5 min the nucleofection solution may have negative outcomes on cellular viability within the cell.
25. Prewarmed/sterile RPMI media supplemented with 1 % BSA, 1 % L-glutamine, and 1 % penicillin/streptomycin should be equilibrated in a 37 °C incubator for 30 min prior to adding the transfected vessel. In some cases the removal of antibiotics may have favorable outcomes on vessel survival during recovery in cell culture incubator. Recovery times will vary for different types of blood vessels. For TDA, 18 h is a sufficient time point to use vessels for pressure myography experiments. Additionally, the maximal knockdown of certain gene products may occur earlier than 18 h and experimental validation is necessary to show that shorter recovery times do not affect vessel functionality. In this case, measuring an experimental output following the transfection protocol without siRNA or with a negative control scrambled siRNA would be the appropriate control.

Acknowledgements

This work was supported by National Institutes of Health grants (HL088554) and HL120840 and National Institutes of Health training grants (HL007284) (BEI) and by a University of Glasgow, Lord Kelvin Adam Smith Fellowship (SRJ).

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