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siRNA-mediated silencing of peroxisomal genes in mammalian cells

Tina A. Schrader, and Michael Schrader*

College of Life and Environmental Sciences, Biosciences, University of Exeter, EX4 4QD Exeter, Devon, UK

*Address all correspondence to Michael Schrader, College of Life and Environmental Sciences, Biosciences, University of Exeter, Geoffrey Pope Building, Stocker Road, Exeter EX4 4QD, UK

E-mail: m.schrader@exeter.ac.uk

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Summary

RNAi technologies are a valuable tool in the identification and investigation of proteins which are involved in peroxisome biogenesis and function. Small interfering RNA (siRNA) has developed into the most commonly used RNAi tool for the induction of transient, short-term silencing of protein coding genes. Although siRNA can induce gene knockdown in a variety of mammalian cell lines, their utility is limited by efficient uptake of synthetic oligonucleotides into the cells. Here, we describe different transfection methods which have been successfully used by us to silence peroxisomal genes in a variety of cell lines, including primary human skin fibroblasts, which are usually difficult to transfect.

1 Introduction

Small (or short) interfering RNA (siRNA) represents a class of double-stranded RNA molecules, which specifically target a particular mRNA for degradation, thus resulting in gene silencing and knockdown of protein expression (*1-3*). siRNA acts via the RNA interference (RNAi) pathway, where it binds to protein complexes such as Dicer (which dices up siRNA into smaller fragments) and RISC (RNA-induced Silencing Complex), which leads to specific RNA cleavage and degradation (*1-3*). siRNA can act in RNAi-related pathways as an antiviral mechanism, but synthetic siRNAs can also induce RNAi in mammalian cells (*4*). Therefore, siRNA has developed into the most commonly used RNAi tool for the induction of transient, short-term silencing of protein coding genes.

RNAi technologies have been proven to be a valuable tool in the identification and investigation of proteins which are involved in peroxisome biogenesis and function. One illustrative example is the discovery of a role for the dynamin-related protein 1 (DRP1), a large GTPase with mechanochemical properties, in peroxisomal fission (*5-8*). Efficient knockdown of DRP1 in cells of human and rodent origin was achieved with a conserved

DRP1 target sequence corresponding to the coding region 783-803 (*5, 7*) (**Fig. 1**). Remarkably, silencing of DRP1, which localizes to both peroxisomes and mitochondria, resulted in a massive membrane elongation of peroxisomes (and mitochondria) due to a block in organelle division (5, 7) (**Fig. 1**). This characteristic peroxisomal morphology was later observed in patient fibroblasts and led to the discovery of DRP1 deficiency, the first member of a new group of disorders with defects in peroxisomal and mitochondrial division (*9, 10*). Although siRNA can induce gene knockdown in a variety of mammalian cell lines, their utility is limited by efficient uptake of synthetic oligonucleotides into the cells. Here, we describe different transfection methods which have been successfully used by us to silence peroxisomal genes in a variety of cell lines, including primary skin fibroblasts, which are usually difficult to transfect.

2 Materials

All reagents and equipment used to maintain and process living cells need to be sterile, and appropriate aseptic techniques and practices should be applied at all times.

2.1 Mammalian Cells and siRNA

1. Mammalian cell line of interest, here: human skin fibroblasts, HepG2 (human hepatoblastoma cells) (American Type Culture Collection ATCC HB-8065), COS-7 (African green monkey kidney cells) (American Type Culture Collection CRL-1651).
2. siRNA for silencing of candidate genes/proteins in mammalian cells, here: DRP1, ACBD5 (*see* **Notes 1** and **2**).

2.2 Cell Culture Equipment

1. Class II Biological Safety Cabinet/Tissue Culture Hood (*see* **Note 3**).

2. Humidified CO_2 incubator (95% air, 5% CO_2, 37°C).
3. Inverted light microscope (phase contrast).
4. 37°C water bath.
5. Mammalian cell counter.
6. Vacuum aspiration system.
7. Table top centrifuge equipped with a swing-out rotor for 15-ml conical tubes.
8. Microporator Neon Transfection System, Invitrogen
9. Electroporator BTX ECM 600 (630) (Harvard Apparatus), BTX, Holliston, MA, USA

2.3 Cell Culture Media, Buffers, and Reagents

1. Complete growth medium (for human skin fibroblasts and COS-7 cells): Dulbecco's modified Eagle's medium (DMEM), high glucose (4.5 g/L) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM Glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin (store at 4°C). For HepG2 cells: Minimum essential medium (MEM-Eagle, Earle's salts) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM Glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin (store at 4°C).
2. Phosphate-buffered saline (1×PBS) (without Ca^{2+} and Mg^{2+}): 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, 2.5 mM KCl, pH 7.4 (store at 4°C).
3. TrypLE™ Express solution (1×) (Gibco) (store at 4°C) (*see* **Note 4**).
4. 70% (v/v) ethanol.
5. Clean and sterile glass coverslips (19 mm ∅, 0.13-0.17 mm thickness) (*see* **Note 5**).
6. Metal tweezers

2.4 Transfection with siRNA

1. Neon Transfection Kit, Invitrogen. Store buffers at 4ºC.

2. HEPES-buffered saline solution (HBS): 21 mM Hepes, 137 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 6 mM dextrose, pH 7.15. Prepare fresh for transfection.
3. 4 mm gap electroporation cuvettes (Molecular Bioproducts, Inc., San Diego, CA, USA).
4. Lipofectamine 3000, Invitrogen. Store at 4ºC.
5. Opti-MEM, Life Technologies. Store at 4ºC.
6. siRNA in sterile, RNase/DNase-free water or buffer supplied by manufacturer (50 – 100 μM stock).

2.5 Controls

Controls are an essential part of every siRNA experiment. It is recommended to add a positive control, a negative control and an untreated control. A well-characterized positive control allows ensuring the experimental set-up (e.g. transfection method, cell line used) is sufficient to achieve effective silencing. We usually include DRP1-specific siRNA as a positive control, which results in a characteristic elongation of peroxisomes (*11-14*) (**Fig. 1**). This morphological alteration can easily be detected by immunofluorescence using peroxisomal marker proteins (**Fig. 1**). Negative controls help to distinguish sequence-specific effects from the effects of experimental conditions on cellular responses. An untreated control serves as a useful reference for cell and organelle phenotypes and gene expression levels. To exclude off-target effects, different and optimised siRNAs can be used (*see* **Note 2**).

Place Fig. 1 here

3 Methods

3.1 Cell Culture

1. Perform all cell culture related work in a Class II Biological Safety Cabinet/Tissue Culture Hood and disinfect the work surface and materials (e.g., pipettes) with 70% (v/v) ethanol.
2. Grow mammalian cells of choice (here, human skin fibroblasts, HepG2, or COS-7 cells) in complete growth medium (10 cm ∅ cell culture dishes) in a humidified CO_2 incubator (95% air, 5% CO_2, 37°C).
3. Refresh the cell culture medium every 2-3 days.
4. Split the cells before they reach 100% confluency (*see* **steps 4** to **14**).
5. Pre-warm 1×PBS, TrypLE™ Express solution, and complete growth medium to 37°C.
6. Remove all medium from the cell culture dish with a Pasteur pipette by vacuum aspiration and wash the cells once with 4 mL of 1×PBS.
7. Add TryplExpress solution to the cells and gently tilt to cover the surface (1 mL/10 cm dish).
8. Incubate the cells for 2-5 min at 37°C.
9. Upon detachment, harvest the cells in complete growth medium (10 mL/10 cm dish).
10. Carefully resuspend the cells by pipetting the cell suspension 2-3 times up and down and further detach remaining cells from the surface of the dish.
11. Transfer the cells to a 15-mL conical tube and take an aliquot of the suspension for cell counting.
12. Pellet the cells by centrifugation (500×*g*, 3 min at RT).
13. Resuspend the cell pellet in 10 mL of complete growth medium.
14. For maintenance, transfer the required amount of cells (approx. 5×10^5 cells) to a new 10 cm cell culture dish containing 10 mL of complete growth medium (*see* **step 4**).

15. Incubate the cells in a humidified CO_2 incubator (95% air, 5% CO_2, 37°C).

3.2 Transfection of siRNA into human skin fibroblast using microporation

1. Split an appropriate amount of cultured human fibroblasts 24 hours before transfection and seed 4×10^5 cells per 10 cm ∅ culture dish. This amount of cells is required for one transfection using the 100 µl Neon tip (*see* **Note 6**). Prepare additional 10 cm dishes as required. Always include a control. It is advisable to prepare 1-2 extra dishes as a backup (4×10^5 cells per dish).
2. On the day of transfection, add 4 ml complete growth medium (without antibiotics!) into a 6 cm ∅ culture dish and place in the incubator (*see* **Note 7**). For each microporation, a 6 cm ∅ dish is required. For analysis by immunofluorescence, add 4 sterile, clean glass coverslips to the dish prior to adding the medium using sterile tweezers. We routinely use round coverslips (19 mm ∅, 0.13-0.17 mm thickness). Coverslips are not required for analysis by immunoblotting.
3. Harvest the cells by trypsination, pool them and centrifuge at 500×*g* for 3 min.
4. Aspirate the supernatant, resuspend the cell pellet in 1×PBS, and centrifuge as above.
5. During centrifugation, place the Neon Microporation device in the biological safety cabinet. Fill a Neon tube with 3 ml of electrolyte buffer E2 and insert the tube into the Pipette station. Set the pulse conditions (here, 1400 V, 20 ms pulse width, 1 pulse) on the device (*see* **Notes 8 and 9**).
6. Aspirate the supernatant, and resuspend the cell pellet in R-buffer (add 110 µl R-buffer per 4×10^5 cells) (*see* **Note 10**). Gently transfer the cells into a sterile 1.5 ml microcentrifuge tube.
7. Add 100 nM siRNA into another sterile 1.5 ml microcentrifuge tube (*see* **Note 11**)
8. Gently add 110 µl of cell suspension (in R-buffer) and pipette 1-2 times up and down.

9. Mount a 100 µl Neon tip onto the Neon pipette.
10. Immerse the tip into the cell-siRNA mixture and slowly aspirate 100 µl of the sample. Avoid generating air bubbles in the tip.
11. Insert the pipette into the E2 buffer-containing tube in the pipette station, and press start on the touch screen.
12. After delivery of the electric pulse, remove the pipette from the pipette station and immediately transfer the cells from the tip to the 6 cm dishes containing the prewarmed growth medium (without antibiotics) (*see* **Note 12**).
13. Gently move the dish horizontal and vertical to evenly distribute the cells (*see* **Note 13**).
14. Incubate the cells in a humidified CO_2 incubator (95% air, 5% CO_2, 37°C) for 72 hours to allow efficient silencing (*see* **Note 14**).
15. Discard the Neon tip in an appropriate biological hazardous waste container and repeat steps 7 to 15 for the remaining cell-siRNA mixtures (control siRNA and other siRNA samples) (*see* **Note 15**).
16. Cells are usually assayed for protein level and peroxisome morphology by indirect immunofluorescence and standard immunoblotting of cell lysates (**Fig. 2**).

Place Fig 2 here (or alternatively at the end before the Notes)

3.3 Transfection of siRNA into mammalian cells using electroporation

1. Split an appropriate amount of cultured COS-7 or HepG2 cells 48 hours before transfection and grow to 80-90% confluency on an area of 75 cm^2/flask or 10 cm ∅ culture dish.

2. On the day of transfection, harvest the cells (1×10^7) by trypsination and centrifuge at $500\times g$ for 3 min (*see* **Note 16**).
3. Resuspend the cell pellet in 10 mL HEPES-buffered saline solution (HBS), centrifuge as above and resuspend the cell pellet in 0.5 mL HBS.
4. Transfer the sample to a 0.4 cm gap, sterile electroporation cuvette containing 20-50 µl of siRNA (20 µM) and mix quickly by pipetting.
5. Place the cuvette in the electroporator and perform the electroporation using the following settings: 230 V (use 250 V for HepG2 cells), 1500 µF, 129 Ω, 25–30 ms duration (*see* **Note 17**).
6. After electroporation, immediately resuspend the cells in 1 mL of complete growth medium. Discard the viscous foam produced during electroporation. Dilute in pre-warmed growth medium and plate the cells in 6 cm ∅ dishes (*see* **Note 18**). For analysis by immunofluorescence, add 4 sterile, clean glass coverslips to the dish prior to adding the medium using sterile tweezers. We routinely use round coverslips (19 mm ∅, 0.13-0.17 mm thickness). Coverslips are not required for analysis by immunoblotting. Coating of the cover slips/dishes (e.g. with collagen) can improve attachment.
7. Cells are usually assayed for protein level and peroxisome morphology after 2-3 days by indirect immunofluorescence and standard immunoblotting of cell lysates (*see* **Note 19**) (**Figs. 1, 2**).

Using the transfection protocol described, we routinely obtain transfection efficiencies between 70 and 80%. Re-transfection is not required.

3.4 Transfection of siRNA into mammalian cells using lipofection

1. Split an appropriate amount of cultured HepG2 cells 24 hours before transfection and seed 250,000 cells on collagen-coated 6 cm ∅ culture dishes. For analysis by immunofluorescence, add 4 sterile, clean glass coverslips to the dish prior to adding the growth medium.
2. On the day of transfection, add 3.3 µL siRNA (from 50 µM stock) to 250 µL Opti-MEM in a 1.5 mL microcentrifuge tube. In a second microcentrifuge tube, add 17 µL Lipofectamine 3000 to 250 µL Opti-MEM. Pipet the siRNA mix to the Lipofectamine mix. Gently pipet up and down 3-4 times and incubate for 5 min. at room temperature. The mixture is sufficient for transfection of one 6 cm ∅ culture dish.
3. Meanwhile, wash the cells once with PBS and add 3.5 mL of fresh complete growth medium.
4. Add the siRNA/Lipofectamine mix dropwise to the cells and incubate in a humidified CO_2 incubator (95% air, 5% CO_2, 37°C) for 72 hours to allow efficient silencing.
5. Cells are usually assayed for efficient silencing by indirect immunofluorescence and standard immunoblotting of cell lysates (**Figs. 1, 2**). Using the transfection protocol described, we routinely obtain transfection efficiencies between 70 and 80%. Electroporation (*see* **3.3**) or microporation (*15*) are alternative transfection methods for HepG2 cells.

4. Notes

1. Gene silencing in mammalian cultured cells can be achieved by DNA-directed RNAi, in which a DNA vector is used to express short hairpin RNAs, or by directly introducing small, interfering RNA duplexes (siRNA) into target cells. Chemically synthesized small oligonucleotide siRNA duplexes are usually easier to transfect than DNA-based vectors that express siRNA.

2. Synthetic siRNAs are most commonly generated through solid-phase chemical synthesis methods (e.g. 2'-ACE chemistry) providing highly pure, stable, and readily modified siRNAs. Several manufacturers offer support in siRNA design and provide optimized and validated siRNAs and appropriate controls. siRNAs can also be used for knockdown of non-protein coding genes, such as long noncoding RNAs.
3. Follow the biosafety and GMO guidelines of your institution.
4. TrypLE™ Express (12604013, Gibco) is an animal origin-free, RT-stable, recombinant enzyme suitable for the dissociation of a wide range of adherent mammalian cells. It cleaves peptide bonds on the C-terminal sides of lysine and arginine, and is a direct replacement for trypsin. Its high purity increases specificity and reduces damage to cells that can be caused by other enzymes present in some trypsin extracts. Alternatively, Trypsin/EDTA solution (1x) can be used: 0.05% (w/v) trypsin, 0.68 mM EDTA, 5.5 mM glucose, 137.93 mM NaCl, 5.36 mM KCl, 6.9 mM $NaHCO_3$ (store at -20°C).
5. For sterilisation, glass coverslips are put in a glass petri dish, wrapped in tin foil and dry-sterilised for 6 h at 180°C.
6. Splitting the cells 24 hours prior to transfection is important to avoid stress due to high cell density. Furthermore, cell counting on the day of transfection is not required anymore and will reduce the handling time of the cells during the transfection procedure.
7. The addition of antibiotics to the growth medium can drastically reduce the viability of freshly transfected cells.
8. A detailed instruction manual of how to use the Neon device can be downloaded from the supplier's website (http://tools.thermofisher.com/content/sfs/manuals/neon_device_man.pdf).
9. Pulse conditions for other cell types need to be optimized. Further information is provided on the supplier's website (https://www.thermofisher.com/be/en/home/life-science/cell-

culture/transfection/transfection---selection-misc/neon-transfection-system/neon-protocols-cell-line-data.html).

10. We use 10% more R-buffer (110 µl instead of 100 µl) to compensate for pipetting errors and to avoid aspiration of air.
11. Use conical 1.5 ml microcentrifuge tubes instead of round bottom 2 ml microcentrifuge tubes to avoid generation of air bubbles.
12. In case of an electric spark during the electric pulse (due to potential air bubbles), discard the tip with the cells and repeat the microporation using the backup cells.
13. Do not swirl, as this will lead to the accumulation of the cells in the center of the dish.
14. Based on our experience, a change of the growth medium during the 72 hours incubation period is not required.
15. The Neon pipette tips and tubes can be regenerated and reused as described (*16*), thereby reducing the cost of microporation at least 10-fold.
16. Electroporation can result in high transfection rates and usually requires non-adherent cells (e.g. suspension cultures or trypsination of adherent cells). However, a dish-electrode for in-dish transfection of adherent cells is as well available (BTX Harvard Apparatus).
17. Electroporation of siRNA typically requires optimization of various parameters that affect cell uptake and cell viability (e. g., electroporation medium, cell number, voltage, capacitance, resistance). It is advisable to test siRNA efficiency at various time points after transfection.
18. Cells can also be plated in 6-well plates (2×10^5 cells/well).
19. Analysis of peroxisome morphology and siRNA efficiency is facilitated by the use of cells stably expressing a fluorescent fusion protein bearing a peroxisomal targeting signal (e. g., GFP-PTS1, DsRed-PTS1) (*7*).

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Figure legends

Figure 1. siRNA-mediated silencing of the dynamin-like fission GTPase DRP1/DLP1 induces peroxisomal membrane elongation. COS-7 cells stably expressing a fusion of GFP and a peroxisomal targeting signal (GFP-PTS1) were transfected with DRP1 siRNA using lipofection and processed for immunofluorescence with anti-DRP1 antibodies 48 hours after transfection. (**A, B**) Localisation of peroxisomes (green) and DRP1 (red). DRP1 is efficiently silenced in cells that took up the DRP1 siRNA (**A**) (asterisks). Note the untransfected, non-silenced cell on the right, which shows strong labeling for DRP1 in the cytoplasm and contains spherical instead of elongated peroxisomes. (**B**) Higher magnification view of elongated, constricted peroxisomes (arrows) after efficient silencing of DRP1. DRP1 is not

efficiently silenced in the cell on the left (x). N, nucleus. (**C**) Immunoblots of peroxisomal fractions isolated from controls (Con) and cells transfected with DRP1 siRNA using anti-PMP70 (peroxisomal membrane protein) and anti-DRP1 antibodies. Equal amounts of protein (PMP70, 10 µg/lane; DRP1, 45 µg/lane) were loaded onto the gel (taken from (*7*) with kind permission of the Company of Biologists). Bars, 10 µm.

Figure 2. siRNA-mediated silencing of ACBD5, a peroxisomal acyl-CoA binding domain protein, in patient skin fibroblasts. (**A, B**) Human skin fibroblasts from a patient with a defect in Mff, a membrane adaptor for DRP1 at peroxisomes (and mitochondria) (*17-19*) were transfected with ACBD5-specific or control siRNA using microporation and processed for immunofluorescence with anti-ACBD5 and anti-catalase (aCAT) antibodies 72 hours after transfection. (**A**) ACBD5 co-localises with the peroxisomal marker protein catalase in controls cells. Note that peroxisomes in Mff-deficient cells are highly elongated due to a block in membrane fission. (**B**) ACBD5 is efficiently silenced in cells that took up the ACBD5 siRNA. Occasionally, some residual ACBD5 is visible at peroxisomes. (**C**) Immunoblots of cell lysates from controls (scrambled siRNA) and cells transfected with ACBD5 siRNA using anti-ACBD5 and anti-tubulin antibodies. Equal amounts of protein were loaded onto the gel. Tubulin served as a loading control. Bars, 10 µm.