6

Nasal Delivery of siRNA

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Summary

The intranasal administration of siRNA has opened new vistas in drug delivery and respiratory therapy. In this strategy, synthetic siRNA with or without chemical modifications can be applied intranasally. Various delivery vehicles have been tested and optimized. With a few exceptions, all promote significant uptake of siRNA into the lung tissue and offer protection against respiratory viruses such as respiratory syncytial virus (RSV), parainfluenza virus (PIV), and influenza virus. No major adverse immune reaction has been encountered. Nasally applied siRNA remains within the lung and does not have systemic access, as judged by its absence in other major organs such as the lung, liver, heart, and kidney. We provide techniques for using the nose as a specific route for siRNA delivery into the lung of laboratory animals, which has enormous potential for clinical applications.

Key Words: siRNA; intranasal; RNAi; antiviral; RSV; parainfluenza; influenza.

1. Introduction

Small interfering siRNAs (siRNAs) have emerged as a promising tool to downregulate gene expression in worms, plants, animals, and humans ***(1–8)*** with great potential to serve both as a research tool and as a therapeutic modality for targeted knockdown of disease-causing genes. Several studies have demonstrated the possibility of using siRNA to treat viral infections ***(7–17)***, inflammatory diseases ***(18,19)***, cancer ***(20,21)***, and genetic ***(22,23)*** and ocular ***(16,24–27)*** diseases. The success of siRNA in RNAi is due to its complementarity to its target mRNA. RNAi includes several steps (*see* **Fig. 1** in **Chapter 4**) ***(28)***. In brief, the antisense strand of the siRNA, as part of

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Fig. 1. Nasal instillation in mice. From left: gloved hand, anesthetized white BALB/c mouse, pipette tip being used for instillation. Note that the proper angle of the tip relative to the nostril is important to prevent spillage of solution and nosebleed.

the RNA-induced silencing complex (RISC), serves as the guide strand to engage the complementary mRNA that is then cleaved by the endonuclease component of RISC, namely Argonaute 2, resulting in gene silencing. Perhaps the most promising area of siRNA application is *in vivo*, which allows a close examination of gene function in living organisms and combating of viral infections. Application of RNAi *in vivo* is, however, still in its early stages, specific delivery being a significant problem. The two major routes of siRNA delivery are systematic and local [e.g., ocular ***(25–27)***, cerebral (***29,30***), peritoneal (***31),*** and nasal ***(7,8,10,32)***].

The nasal route presents an interesting regimen since it allows noninvasive means of delivery of siRNA to lung cells, which is experimentally and therapeutically useful. It can also be useful in treating such debilitating respiratory tract disorders as chronic obstructive pulmonary disease (COPD), cystic fibrosis, asthma, as well as many viral infections of the lung.

2. Materials

We have successfully used the reagents described below, but various equivalents are available commercially that can be optimized. We also list a number of popular Web sites that offer useful advice on various aspects of RNAi **(Table 1)**.

Table 1
Useful Web Sites

URL	Content
www.ambion.com	Predesigned and custom siRNA, siRNA design algorithm, transfection reagents for *in vivo* and *in vitro* siRNA transfection
www.dharmacon.com	Custom siRNA design at the siDesign™ Center, large selection of predesigned siRNA targeting most human, mouse, and rat genes, transfection reagents
www.invitrogen.com	Custom siRNA using the BLOCK-iT™ RNAi Designer algorithm, Stealth™ Select RNAi (predesigned sequences to the majority of the human, rat, and mouse genes)
www.mirusbio.com	Transfection reagents for *in vivo* and *in vitro* siRNA delivery, reagents for siRNA localization and tracking, fluorescent delivery controls

2.1. RNA Work

1. DEPC-treated water (Sigma-Aldrich, St. Louis, MO).
2. RNase-free ART® aerosol resistant pipette tips (Molecular BioProducts, San Diego, CA).
3. RNase-free microfuge tubes (Ambion, Austin, TX).
4. 9.5 *M* of ammonium acetate.
5. 95% and 100% Ethanol.
6. 2′-Deprotection buffer: 100 m*M* acetic acid-TEMED, pH 3.8 (Dharmacon, Lafayette, CO).
7. siRNA buffer: 20 m*M* KCl, 6 m*M* HEPES-KOH, pH 7.5, 0.2 m*M* $MgCl_2$.

2.2. Animals and Anesthesia

1. BALB/c mice, 8–10 weeks old, weighing 16-20 g (Charles River Laboratories, Wilmington, MA).
2. 5 mg/mL of sodium pentobarbital (Nembutal™).
3. 25-gauge single-use needles (VWR, Westchester, PA).
4. 1-cc single-use syringes with BD Luer-Lok tip (VWR).

2.3. siRNA Transfection

1. TransIT-TKO siRNA transfection reagent (Mirus Bio Corporation, Madison, WI).
2. Opti-MEM I Reduced Serum Medium (Gibco™, Invitrogen Corporation, Carlsbad, CA).
3. RNase-free gel-loading microcapillary tips (VWR).
4. RNase-free microfuge tubes (Ambion).

3. Methods

3.1. siRNA 2′-Deprotection, Annealing, and Desalting

1. Add 200 µL of 2′-deprotection buffer to tube containing 0.1 µmol of 2′-ACE protected, single-stranded RNA.
2. Combine the volumes of complementary strands of RNA, vortex for 10 sec, and centrifuge for 30 sec.
3. Heat the mixture at 60 °C for 45 min.
4. Remove from heat and centrifuge briefly, 5–10 sec.
5. Allow solution to cool to room temperature over 30 min.
6. Add 40 µL of 9.5 *M* ammonium acetate and 1.5 mL of 100% ethanol to the 400 µL of siRNA duplex solution, and vortex.
7. Place the tubes at –20 °C for >16 h or at –70 °C for 2 h.
8. Centrifuge at 14,000 x g for 30 min at 4 °C.
9. Carefully remove the supernatant away from the pellet.
10. Rinse the pellet with 200 µL of ice-cold 95% ethanol.
11. Dry under vacuum using Speed-Vac.
12. The dry pellet can be stored at –20 °C until use or resuspended in an appropriately buffered solution (20 m*M* of KCl, 6 m*M* of HEPES-KOH, pH 7.5, 0.2 m*M* of $MgCl_2$).

3.2. siRNA–Vehicle Complex Formation

Perform siRNA complex formation immediately before nasal administration. Determine the optimal siRNA amount by titrating from 3 to 15 nmol per mouse (*see* **Note 2**).

1. In a sterile, RNase-free plastic tube, add 35 µL of Opti-MEM reduced-serum medium.
2. Add 5 µL of the TransIT-TKO transfection reagent into the tube containing the Opti-MEM medium.
3. Mix thoroughly by vortexing for 10 sec.
4. Incubate at room temperature for 10 min.
5. Add desired amount of siRNA in 1 µL of siRNA buffer to the diluted TransIT-TKO reagent.
6. Carefully mix by gentle pipetting.
7. Incubate at room temperature for 20 min.

3.3. Animal Anesthesia

Prior to nasal administration of siRNA, the mouse must be anesthetized to minimize any pain or discomfort. Nembutal is administered by intraperitoneal (IP) injection. The recommended drug dosage for mice is 50 mg/kg (*see* **Note 1**).

1. Gently lift the mouse by the tail and place it on a cage lid.
2. Grip the loose skin of the neck to immobilize the head of the animal.

3. With the head immobilized, extend the tail to draw the skin tight over the abdomen by gripping the tail with your little finger.
4. The animal should be held in a head-down position.
5. Disinfect injection site.
6. Insert the hypodermic needle into the lower right or left quadrant of the abdomen.
7. Place animal back into the cage and wait until anesthesia takes effect.
8. The animal is ready for siRNA administration when no voluntary movement is observed.

3.4. siRNA Administration

1. Place anesthetized animal on a lab towel facing up (**Fig. 1**).
2. With the mouse's head immobilized, insert microcapillary tip containing siRNA/transfection reagent complexes into the nostril.
3. Instill solution slowly over a 2–3-min period, allowing the mouse to breathe the liquid in.
4. Place animal back into the cage and monitor for at least 45 min to avoid depression of cardiac and/or respiratory functions.
5. Test for the desired RNAi effect at appropriate intervals. For antiviral studies, instill virus through the nostril as well (*see* **Notes 3** and **4**). For human RSV, which does not infect mice well, use 10^7–10^8 virus particles per animal, and measure standard lung titer assay and/or clinical symptoms (such as body weight, respiration rate, etc.).

4. Notes

1. We have described a simple method of nasal delivery of siRNA in the mouse model, but it can be successfully scaled up or down to use in other laboratory animals.
2. To diminish any possible toxic effect of delivery reagents, siRNA can be introduced "naked," i.e., without transfection reagent, which exhibits about 70–80% activity of the reagent-complexed siRNA. It is best not to use polyethyleneimine (PEI). Although PEI is often used to form complexes with nucleic acids, mice apparently do not tolerate PEI through the nose. In our attempts, essentially all mice died within minutes of inhalation of the PEI–siRNA complex.
3. Administration of excessive liquid will "drown" the mouse and cause death. Try to keep the total volume under 45 μL in routine application, although up to 100 μL may be tolerated (*see* **Chapter 11**).
4. The procedure also works in an aerosolized application. We have used a small homemade enclosure in which the anesthetized mouse is placed and the siRNA complex (made as in **Section 3.2**) is sprayed in using a handheld nebulizer (the common type used as an inhaler by asthmatics). In this method, a larger amount of siRNA is needed because most of the mist is wasted and a small fraction is inhaled by the animal. To optimize cost versus benefit, it is recommended that various amounts of mist and duration of exposure are tried for a given enclosure

volume. If this method is to be routinely used, a commercial motorized nebulizer can be easily optimized. It is best to check with the local pediatric department for the exact model, vendor, and usage. Modify the system by removing the plastic face cup (or mask) at the delivery end and insert the tube directly into the mouse enclosure. Reagent cost can be reduced by designing a smaller face cup to snugly fit the mouse's nose area.

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