molecular pharmaceutics

reviews

Inhalable siRNA: Potential as a Therapeutic Agent in the Lungs

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Received April 27, 2007; Revised Manuscript Received April 1, 2008; Accepted April 5, 2008

Abstract: RNA interference (RNAi) is gaining increasing popularity both as a molecular biology tool and as a potential therapeutic agent. RNAi is a naturally occurring gene regulatory mechanism, which has a number of advantages over other gene/antisense therapies including specificity of inhibition, potency, the small size of the molecules and the diminished risk of toxic effects, e.g., immune responses. Targeted, local delivery of RNAi to the lungs via inhalation offers a unique opportunity to treat a range of previously untreatable or poorly controlled respiratory conditions. In this timely review we look at the potential applications of RNAi in the lungs for the treatment of a range of diseases including inflammatory and immune conditions, cystic fibrosis, infectious disease and cancer. In 2006 Alnylam initiated the first phase 1 clinical study of an inhaled siRNA for the treatment of respiratory syncytial virus. If its potential as a therapeutic is to be realized, then safe and efficient means of targeted delivery of small interfering RNA (siRNA) to the lungs must be developed. Therefore in this review we also present the latest developments in siRNA delivery to airway cells *in vitro* and the work to date on *in vivo* delivery of siRNA to the lungs for the treatment of a range of diseases.

Keywords: siRNA; lungs; inhalation; respiratory disease

Introduction

RNA Interference. In 2006 Andrew Z. Fire and Craig C. Mello were awarded the Nobel Prize for their discovery of RNA interference (RNAi). This pathway is involved in cellular defense against viral invasion and transposon expansion and represents a unique form of post-transcriptional gene silencing.[1] It is also a cost-effective molecular biology tool for the determination of gene function, signaling pathway analysis, RNAi mechanistic studies and target validation and shows tremendous potential for diagnostics and therapeutics.

RNAi is an evolutionarily conserved process observed in the majority of eurkaryotes studied to date. The functional unit of the pathway is small interfering RNA or siRNA.[2] siRNA can be specifically synthesized and introduced into a cell to induce gene silencing. As this methodology exploits a naturally occurring pathway, it differs from other silencing technologies such as antisense oligonucleotides. In nature, RNAi is initiated when the cell encounters ectopic double stranded RNA (dsRNA), e.g., viral RNA, transposon or microRNA (miRNA). In the cytoplasm the RNase III-like protein dicer cleaves dsRNA from miRNAs or replicating viruses into siRNAs of 19−25 bases in length (Figure 1). The siRNA is then incorporated into the multiprotein RNA-induced silencing complex (RISC), which unwinds the duplex producing two strands; one strand (passenger) is discarded while the other (guide) can independently guide targeted mRNA recognition. The binding of siRNA results in a site-specific cleavage of the mRNA thereby silencing the message. The released cleavage products are degraded, and the siRNA:RISC complex is free to find another mRNA target. Degrading mRNA results in a profound reduction in the levels of the corresponding protein without altering the

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10.1021/mp070048k CCC: $40.75
Published on Web 05/21/2008

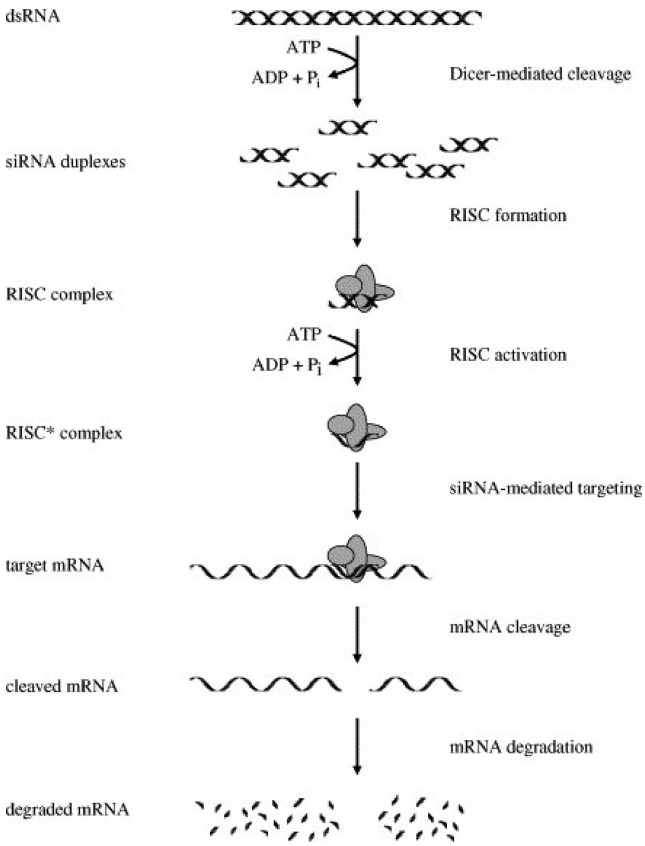

Figure 1. Mechanism of RNA interference (RNAi) in mammalian systems. Reprinted with permission from ref 62. Copyright 2005 Elsevier B.V.

DNA. RNAi is therefore a highly promising therapeutic approach for diseases where aberrant protein production is a problem.

RNAi overcomes many of the shortcomings previously experienced with antisense oligonucleotides and aptamers.[3–5] The advantages of siRNA over other gene/antisense therapies are a robust, potent, specific inhibition, a site of action for siRNAs in the cytoplasm, and the diminished risk of toxic effects, e.g., immune responses. It is important to note however that siRNA, and endogenously expressed hairpin siRNA, greater than 30 bp can induce a nonspecific innate immune response involving the activation of interferon.[6,7] However, siRNA is more sensitive to degradation than DNA and may not be completely encapsulated by cationic agents or may form complexes that are too large for efficient gene silencing.[6]

***In Vitro* and *In Vivo* Delivery of siRNA to Lung Cells.** To successfully achieve gene silencing, careful siRNA design and effective delivery to the targeted lung cells is paramount.

siRNA Design. Effective site selection algorithms and several siRNA design guides are currently available.[8–12] The majority of *in vivo* siRNA experiments to date reported the use of 21-mer duplexes with a 19-base central double-stranded region and terminal 2-base 3′ overhangs. This design mimics naturally occurring molecules produced by dicer processing *in vivo*. siRNA can be chemically synthesized or transcribed from a plasmid. In the case of the latter, a DNA insert of approximately 70 bp, encoding for a short hairpin RNA (shRNA) targeting the gene of interest, is cloned into a plasmid vector. The insert containing plasmid can then be transfected into a cell where the shRNA is expressed. The shRNA is rapidly processed by the cellular machinery into 19−22 nt siRNAs, which can then interfere with the expression of the target gene.[13]

Several strategies are being explored to improve siRNA stability *in vivo* based on modifications previously used to improve the stability of antisense molecules. Commonly used modifications to improve stability include phosphorothioate (PS) or boranophosphate modification of the internucleoside linkage. Boranophosphate modifications confer significant nuclease resistance, but synthesis is complex, with modified bases being incorporated using *in vitro* transcription,[14] making site-selective placement difficult. PS modifications are easier to position and will prolong the life of the siRNA when exposed to nucleases. It is important to note, however,

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that while limited PS modification preserves siRNA potency, overmodification may decrease potency and/or increase toxicity.[15–18]

A number of strategies can be used to prevent immune recognition and response, such as the use of delivery agents to avoid retention of siRNA within endosomes. Another common strategy is the modification of the nucleotides of siRNA, such as the replacement of the 2′-hydroxyl uridines with 2′-*O*-methyl uridines.[19,20]

Careful design of siRNA is essential to prevent off-target effects. Nucleic acid−base pairing is highly specific, and mismatches at one or a small number of positions is often sufficient to completely prevent hybridization under physiological conditions.[15,21] It is desirable therefore to synthesize more than one siRNA for each target to control for off-target effects.

siRNA Delivery. Once an effective siRNA has been designed, the next step is to develop an efficient delivery system. There are two main barriers to efficient siRNA delivery to the cells of the lungs. The first is the complex, branched anatomy of the lungs and biomechanical barriers such as the mucus layer covering the airway cells. The second is the airway cell membrane. For efficient gene silencing in the lungs the siRNA must be delivered efficiently to its site of action, be stable, enter the cell and be present in the cytoplasm at a sufficient concentration. Below is a review of the current technologies being exploited for siRNA delivery to the lungs.

Viral Vectors. Both viral and nonviral vectors are being assessed for siRNA delivery to lung cells. Viral vectors have been developed for efficient delivery of siRNA into a range of mammalian cells. Genetic material inserted into the vector can encode for shRNA which efficiently blocks production of a specific protein. Retroviral vectors have been designed to produce siRNA driven by either U6 or H1-RNA promoters for efficient, uniform delivery and immediate selection of stable knockdown in cells. Retroviral systems are effective in most cell lines including primary cells.[22] Adenovirus vectors have been demonstrated to mediate gene silencing in an *in vitro* lung model[23] and to induce RNAi in a range of animal tissues.[24]

Viral based delivery has several disadvantages, however. Immune response to viruses not only impedes gene delivery but can cause severe complications for the patient. Recent well-documented cases, such as the death of Jesse Gelsinger due to complications associated with an adenoviral vector, highlight this problem.[25] Some viral vectors, e.g., lentivirus, may insert their genome at a seemingly random location in the host chromosome, thereby disturbing gene function.[26]

Nonviral Vectors. An alternative to viral vectors is the use of nonviral lipid and polymer-based vectors. While immortalized cell lines can be successfully transfected with nonviral vectors, to date efficient transfection of primary cells has been poor. Therefore finite cell lines or freshly isolated primary cells may be more suitable targets for viral vectors.[27] However, ongoing research into the transfection of primary cells and whole organisms with siRNA using nonviral transfection agents has produced some promising results.

DNA and siRNA are negatively charged, as is the surface of the cell. Therefore using positively charged lipid and polymer based transfection agents can aid in their introduction into the cell by complexing and protecting the negatively charged siRNA and enhancing interactions with the cell surface.[28] Both lipid and polymer based transfection agents have been used successfully to introduce genetic material to cells in culture as well as systemically and locally to animal models.

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Table 1. Vectors Used To Deliver siRNA to Airway Cells in Culture

transfection reagent	cell type	mRNA target
Dharmafect 1	NCI H1299	RASSF1C[54]
Fugene6	A549	Caveolin-1[55]
Lipofectamine	Calu-3	c-erbB2[48]
Lipofectamine 2000	BEAS2B	NFkB (p65)[50]
Lipofectamine 2000	Glc-82	GFP[56]
Oligofectamine	H1703	DVL-3[57]
sIMPORTER	NHTBE	RAR/RXR[58]
siPORT *Neo*FX	A549	GAPDH (Ambion)
TransIT-TKO	CL1-0/CL1-5	Axl[59]

Several commercial lipids have been available for many years for intracellular DNA delivery. However, effective systems for DNA delivery are not always optimal for the delivery of siRNA into cells. A number of transfection agents are currently available that are capable of delivering siRNA intracellularly to lung cell lines (Table 1). Some of these products have been available for some time, such as Invitrogen's Lipofectamine and Lipofectamine 2000, while others have been developed more recently such as Upstates sIMPORTER. More transfection reagents are being produced which are specifically designed to transfect cells with siRNA, including Mirus's TranIT-TKO and Dharmacon's Dharmafect. Novagen has amine- and lipid-based reagents in a single formulation called RiboJuice siRNA Transfection Reagent, which is designed to target a wide range of mammalian cell lines, including lung cells.

The transfection efficiency depends on a number of factors: cell type, passage number, cell confluency, the time and manner of formation of siRNA-transfection agent complexes and optimized protocols. The majority of commercial transfection agents are cationic liposome-based systems. Liposomes have also been modified with ligands such as folate to target siRNA delivery to specific cell types, such as a murine lung carcinoma line, or small peptides[29] such as protein transduction domains (PTDs) to enhance cell membrane penetration.[30]

Two important issues, however, limit the usefulness of cationic lipids *in vivo*: their relatively poor transfection efficiency compared to viruses and their *in vivo* toxicity.[31] Newer classes of lipids are being investigated that can fully encapsulate, and not just interact with, nucleic acids, and present a stable, neutral surface lowering the toxicity and improving the stability of the complex.[32]

Cationic polymers have also been assessed for siRNA delivery to lung cells. siRNA combined with polyethylenimine (PEI) administered by injection retro-orbitally has been shown to decrease influenza virus titers in mouse lung.[33,34] As well as an accumulation of siRNA in the lungs after injection, siRNA was also found in the spleen, liver, heart and kidney.[33] The lungs are highly vascularized, containing capillary beds, which are first traversed by intravenous delivered materials, which would explain the rapid uptake of the siRNA by the cells in the lungs.

Modifications of polymers have improved their transfection efficiency. Thomas et al. showed that linear fully deacylated PEI25 and PEI87 were the most effective for the delivery of siRNA to the lungs.[33] The addition of peptides such as HIV-TAT to cationic polyethylene glycol (PEG) PEI polymers, can markedly enhance transfection efficiency in bronchial cells *in vitro*.[35] Kong et al. used nanoparticles, composed of the cationic polysaccharide chitosan to deliver plasmids expressing anti-RSV (respiratory syncytial virus) siRNA to rats. The complex was instilled intranasally one day prior to intranasal infection with RSV. The treatment reduced RSV titers and prevented lung damage and airway hyperreactivity. Chitosan is a polysaccharide which is both mucoadhesive and mucopermeable, two qualities that are advantageous for targeting siRNA delivery to the respiratory tract. In addition it has been shown to be effective for siRNA delivery and gene silencing without toxicity.[36] However, some questions have been raised on the toxicity of both cationic lipids and polymers *in vivo*.[31,37]

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Table 2. *In Vivo* Delivery of siRNA to the Lungs

route	animal model	siRNA target	delivery agent	outcome	ref
intranasal	mouse	Heme oxygenase-1	none	First demonstration of lung-specific siRNA delivery	60
intranasal	mouse	GAPDH	Infasurf	Noninvasive delivery of small inhibitory RNA and other reagents to pulmonary alveoli in mice	43
intranasal	mouse	RSV/PIV	TransIT-TKO	This study was the basis for RNAi taken into the clinic against RSV.	37
intranasal	rat	RSV	chitosan	Reduced RSV titers and prevented lung damage	61
intranasal/ intratracheal	rhesus macaque	SARS	DW5	First demonstration of RNAi effect in a primate lung	44
intranasal	mouse	Influenza A	Oligofectamine	Protection against influenza virus challenge by RNAi *in vivo*	42 42

Targeting siRNA to the Lungs by Inhalation. The most elaborately designed siRNA formulated with the most versatile, well-targeted transfection agents will be of little therapeutic value if it does not reach its target site in the lungs at an adequate dose. The efficiency of inhaled siRNA delivery therefore depends not only on the formulation and transfection agents but also on the device used. The use of aerosols to deliver medication to the lungs has a long history, with the smoking of *Atropa belladonna* leaves being used to suppress coughing in the 16th century.[38] Some of the major obstacles encountered in systemic delivery of siRNA include inefficient targeting to the desired organ and cell type, rapid degradation by nucleases, systemic toxicity and rapid excretion. For delivery to the lungs these problems can be overcome to some extent by local, targeted delivery via inhalation. There are a number of advantages to aerosol delivery including local targeting (e.g., for respiratory disease), a noninvasive method of delivery, immediate availability and decreased systemic toxicity. While there is a limited amount of inhalable siRNA work to date, there is a large body of work conducted on inhalable DNA.[39,40] The introduction of DNA via aerosol has been tested for a number of genes in a number of different animal models. One important factor for successful transfection is the protection of the DNA from the shear forces of nebulization. As well as viral vectors, cationic liposomes and polymers have been used to protect and enhance DNA gene delivery to the lungs.[39] In 1992 Stribling et al. were the first to demonstrate aerosol delivery of cationic liposomes complexed with plasmid DNA, although delivery efficiency was poor.[40] These strategies are also being used for the delivery of siRNA,[41] but certain characteristics of the molecules differ and therefore impact on the method of inhalation. One advantage of siRNA is its efficiency at much lower concentrations. This lower dosing is particularly attractive for inhalation where delivery efficiency can limit the maximum dose deliverable. Special care must be taken to ensure that siRNA stability is maintained during manufacturing and aerosolization.

Early generation inhalers were developed for the delivery of small molecules, and their poor delivery efficiency is inadequate for the delivery of large, expensive molecules such as siRNA. With the advent of the next generation of inhalers, designed primarily for protein delivery, the potential for efficient pulmonary delivery of siRNA is greatly improved. A large number of new devices have been developed to improve drug delivery efficiency to the lungs and minimize biomolecule degradation, and could prove useful for the delivery of siRNA. Advanced liquid-spray systems are being developed by a number of companies including vibrating mesh plate systems, e.g., Aeroneb (Aerogen) and the AERx (Aradigm) system, that incorporate a mechanical force to aerosolize liquids through precision-controlled orifices. Liquid-spray systems are likely to be the first option for siRNA inhalation, but more advanced dry powder systems could potentially be used in the future. The more lengthy and complex formulation required for dry powder inhalers (DPIs) will delay development of siRNA DPIs.

Proof of principle for siRNA therapeutics must ultimately be assessed *in vivo*. With many siRNA therapies still at the preclinical development stage it is important to ensure efficient delivery to the airways of the animal models, often small rodents, thereby providing a robust method with which to assess *in vivo* efficacy of siRNA. To date many of the relevant *in vivo* animal studies looking at targeting siRNA to the lungs have used intranasal instillation with some success.[37,42,43] A summary of the *in vivo* animal studies to date is provided in Table 2. The intranasal delivery method also appears to be effective in primates. Li et al.[44] delivered a siRNA specific for the SARS virus intranasally to rhesus

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macaques, resulting in a decreased viral load, reduced alveoli damage and reduced fever.

The success seen with intranasal instillation in rodent models, which are obligate nose breathers with a very different lung anatomy, may not be easily extrapolated to human studies.[45] Therefore careful choice of efficient inhalation device is paramount. A number of options are available for human trials with pulmonary siRNA delivery. Alnylam's clinical trial using nebulizers for delivery, and technologies, such as microspraying, could be the key to effectively assessing siRNA therapies in humans.[46] The long-term hope would be that some of the newer and more sophisticated devices already on the market, and those still being developed, will be suitable platforms for patient friendly, noninvasive delivery of therapeutic siRNA. Factors such as the dose required, choice of transfection agent, stability, drug aerosolition and therapy duration will influence the delivery method chosen.

Therapeutic siRNA Application in the Lungs

There are a number of potential applications for targeted, local delivery of RNAi to the lungs including the treatment of inflammatory and immune conditions, cystic fibrosis (CF), infectious disease and cancer.

The expression of a large number of genes is known to be altered in cancer, and modifying the expression of these genes is an attractive method of cancer treatment. Therefore, siRNA therapies are currently being studied as potential cancer treatments. The effect of siRNA mediated gene silencing of C-erbB-2 was investigated by Ren et al.[47,48] This gene is overexpressed in some cancers such as breast, ovarian and lung cancer. Its expression is related to enhanced malignancy and metastatic ability, intrinsic chemoresistance and poor prognosis of tumors. Specific siRNA knockdown of C-erbB-2 in Calu-3 cells was found to effectively inhibit C-erbB-2 expression and cell proliferation. It also enhanced Calu-3 cell apoptosis thereby reducing the overall number of transformed cells.

In a similar study, Zhang et al. used siRNA directed against mutant K-ras and determined the antitumor effects of decreasing the levels of this protein in lung cancer cell lines.[49] Results revealed that adenovirus-mediated siRNA can specifically target ras and may be a potential therapeutic to treat human lung cancer. Han et al. determined that fibronectin induces cell proliferation and inhibits apoptosis in the human bronchial epithelial cells BEAS-2B and 16-HBE. These pro-oncogenic effects are mediated by PI3-kinase and NF-κB and can be blocked by the administration of an anti p65 siRNA leading to an antioncogenic effect.[50]

RNAi may also provide opportunities for the treatment of CF, a genetic disorder exhibiting defective chloride transport.[51] The inability to transport chloride is a hallmark

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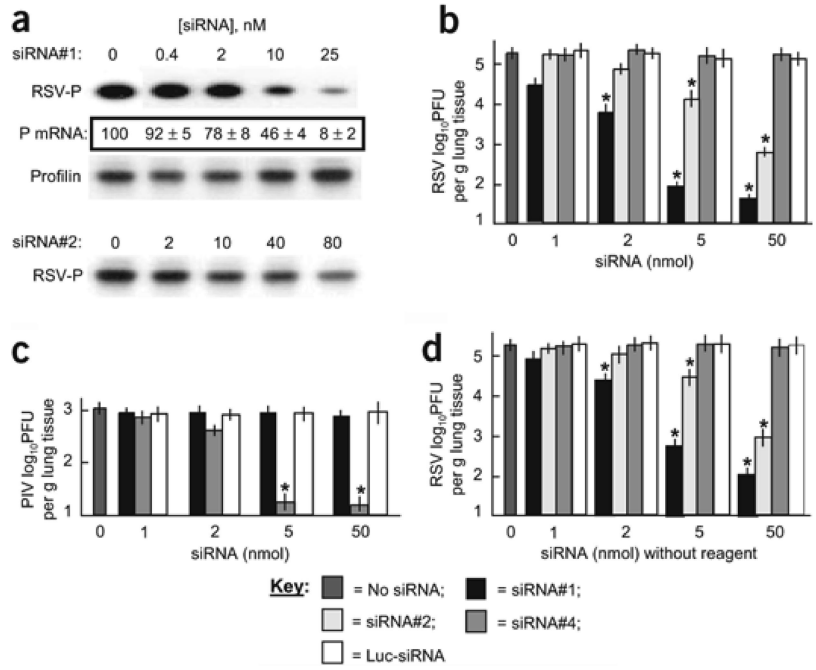

Figure 2. Titration of antiviral siRNAs *ex vivo*. (a) Immunoblot analysis of total proteins of RSV-infected A549 cells (*ex vivo*) with profilin as the internal control. Numbers in the box represent levels of target P mRNA following siRNA treatment, expressed as percentage of untreated levels. In the following three panels, virus was administered 4 h after siRNA. (b) Pulmonary infectious virus in RSV-infected mice ($n = 8$ for each data point). (c) Pulmonary infectious virus in PIV-infected mice ($n = 8$ for each data point). (d) As in panel b except that naked siRNA was administered without any transfection reagent. Asterisks indicate significant inhibition ($P < 0.05$). siRNAs are described in Table 1. Reprinted with permission from ref 37. Copyright 2004 Nature Publishing Group.

of CF resulting in a buildup of thick mucus in several organs, including the pancreas and the lungs, leading to malnutrition, chronic lung infection and damage. A study by Vij et al. has identified and inhibited an overactive protein, valosin containing protein (VCP/pr97), which plays a key role in CF. This protein induces the destruction of the chloride transporters, cystic fibrosis transmembrane conductance regulators (CFTR), in CF patients. In tracheal cell cultures obtained from CF patients it was found that inhibition of VCP/pr97 with siRNA restored the ability of these cells to transport chloride.[51] Anti VCP/pr97 siRNA was compared with the proteasome-inhibiting drug bortezomib. Both drug and RNAi prevented the characteristic interleukin-8 (IL8) mediated inflammation in CF, but protein silencing by the siRNA was superior.[51]

The use of siRNA for the treatment of viral lung infection has also been investigated by a number of groups. Tompkins et al.[42] used unmodified siRNAs specific to influenza viral proteins to protect mice from lethal infection with influenza virus. Influenza virus was coadministered with siRNA, complexed with the cationic lipid Oligofectamine, by direct intranasal infusion in mice. Viral titers in lung tissue were reduced as much as 63-fold using anti-influenza siRNA, compared to controls, with an improved survival. Viruses such as RSV and parainfluenza virus (PIV) have been identified as possible targets for siRNA treatment as there is currently no reliable vaccine or antiviral drug against them. Bitko et al. used unmodified siRNAs to treat RSV and PIV in mice by targeting the viral P gene for both viruses.[37] In this paper the authors show their work from the design of the siRNA through to the treatment of RSV and PIV infected mice. Using one of these algorithms the authors designed and tested siRNA targeting the viral P gene for both viruses (Figure 2). The siRNAs were administered either naked or complexed with the cationic lipid TransIT-TKO by direct intranasal infusion in mice (Figure 2b−d). Treatment with naked siRNA was ~70% as effective as with cationic lipid complexed siRNA treatment. Mice receiving antiviral siRNA displayed decreased lung pathology and overall distress compared to controls. Analysis of lung tissue probed with virus specific antibodies showed that intranasal pretreatment could abolish RSV infection (Figure 3a). The authors demonstrated the presence of the siRNA

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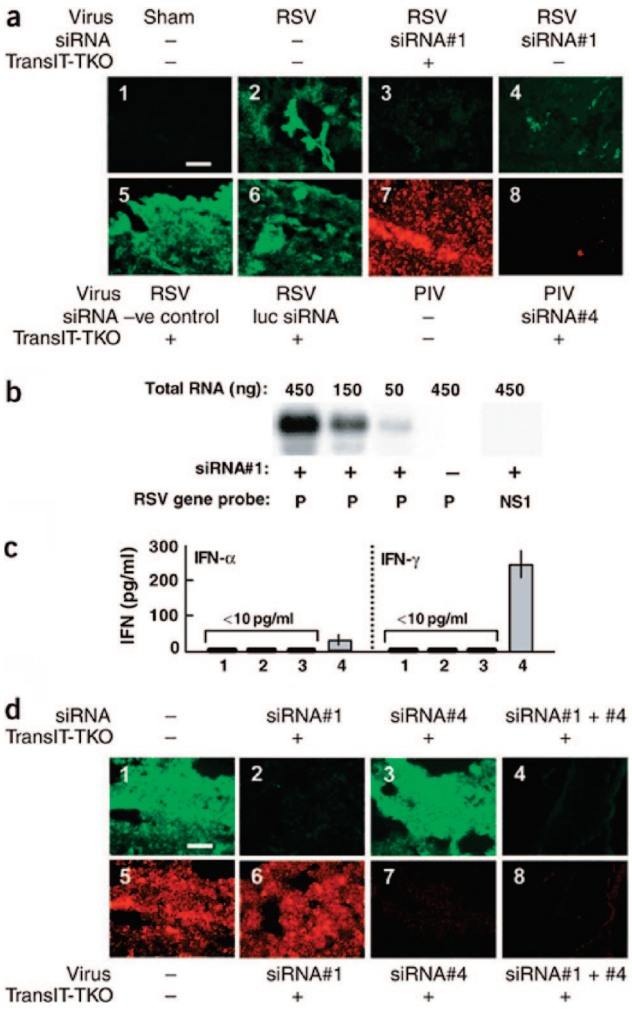

Figure 3. Knockdown of viral antigens in siRNA-treated mouse lung without IFN activation. (a) Virus was administered 4 h after siRNA, and viral antigens were detected by indirect immunohistology of lungs at 4 days after infection (green, RSV; red, PIV). Bar 400 m. (b) Antisense strand of siRNA#1 was detected by northern analysis of varying amounts of total lung RNA 2 days after siRNA administration using labeled RSV P DNA as probe. A probe against RSV NS1 did not react, showing specificity of detection. (c) Intranasal siRNA (10 nmol or 140 g per mouse) did not activate pulmonary IFN- or IFN- above the threshold of detection (10 pg/mL), whereas in control lungs, RSV infection activated type II and low levels of type I. Lanes: 1, siRNA#1; 2, siRNA#4; 3, Luc siRNA; 4, no siRNA but RSV-infected; error bars indicate SEM. Lungs were obtained 2 days after siRNA administration and 4 days after infection ($n = 4$ for each graph). (d) siRNA-mediated inhibition of dual infection by RSV and PIV determined by indirect immunohistology (green, RSV; red, PIV). Virus was administered 4 h after siRNA, and lung tissues were examined at 4 days after infection. Bar, 400 m. Reprinted with permission from ref 37. Copyright 2004 Nature Publishing Group.

in the lungs (Figure 3b) and that the effects seen were not a result of interferon activation (Figure 3c). Interestingly pretreatment with siRNAs for both RSV and PIV prevented infection by both viruses (Figure 3d). The results of this paper indicate the possible potential of siRNA treatment for viral infection of the lungs, as well as for other respiratory conditions.

Sirna Therapeutics, Inc., is exploring the potential of local pulmonary delivery of siRNA for the treatment of respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD). The company is currently using an *in vivo* model to assess airway hyperresponsiveness and is targeting the interlukin-4 receptor (IL-4R). IL-4 is an inflammatory cytokine which drives the airway inflammation underlying airway hyperresponsiveness in asthma.[52] Positive signs have already been observed following local administration of anti-IL-4 siRNA to the lungs, with an 80% decrease in airway hyperresponsiveness being reported.

Alnylam Pharmaceuticals is using the anti-RSV siRNA ALN-RSV01 to treat RSV infection. This virus poses a great risk to young children and immunocompromised individuals. In 2006, a phase I human clinical trial of an inhaled formulation of ALN-RSV01 was initiated to evaluate its safety, tolerability and pharmacokinetics. It was delivered via a nebulizer, advancing previous clinical work where administration was via an intranasal spray. ALN-RSV01 was discovered to be safe and well tolerated in healthy volunteers. Future studies will determine if ALN-RSV01 can reduce RSV infection in infected individuals.[53]

Conclusion

RNAi opens up an exciting new method for the treatment of many respiratory conditions. If its potential is to be harnessed, however, the delivery hurdles that have hampered the development of other gene and antisense therapies must be overcome. Factors such as the dose, the requirement for and choice of transfection agent and the method of inhalation/device will all impact on whether inhalable RNAi will be able to make the move from bench to bedside. It is therefore an exciting area of research, not only for molecular biologists but also for those working in the area of molecular pharmaceutics, to ensure that this technology reaches its full potential.

Acknowledgment. The authors would like to acknowledge the support received from the Irish Health Research Board (HRB).

MP070048K