Manipulation of Cell Physiology Enables Gene Silencing in Well-differentiated Airway Epithelia

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The application of RNA interference-based gene silencing to the airway surface epithelium holds great promise to manipulate host and pathogen gene expression for therapeutic purposes. However, well-differentiated airway epithelia display significant barriers to double-stranded small-interfering RNA (siRNA) delivery despite testing varied classes of nonviral reagents. In well-differentiated primary pig airway epithelia (PAE) or human airway epithelia (HAE) grown at the air–liquid interface (ALI), the delivery of a Dicer-substrate small-interfering RNA (DsiRNA) duplex against hypoxanthine–guanine phosphoribosyltransferase (HPRT) with several nonviral reagents showed minimal uptake and no knockdown of the target. In contrast, poorly differentiated cells (2–5-day post-seeding) exhibited significant oligonucleotide internalization and target knockdown. This finding suggested that during differentiation, the barrier properties of the epithelium are modified to an extent that impedes oligonucleotide uptake. We used two methods to overcome this inefficiency. First, we tested the impact of epidermal growth factor (EGF), a known enhancer of macropinocytosis. Treatment of the cells with EGF improved oligonucleotide uptake resulting in significant but modest levels of target knockdown. Secondly, we used the connectivity map (Cmap) database to correlate gene expression changes during small molecule treatments on various cells types with genes that change upon mucociliary differentiation. Several different drug classes were identified from this correlative assessment. Well-differentiated epithelia treated with DsiRNAs and LY294002, a PI3K inhibitor, significantly improved gene silencing and concomitantly reduced target protein levels. These novel findings reveal that well-differentiated airway epithelia, normally resistant to siRNA delivery, can be pretreated with small molecules to improve uptake of synthetic oligonucleotide and RNA interference (RNAi) responses.

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Introduction

Small-interfering RNA (siRNA)-mediated silencing of genes offers a novel approach for disease treatment. Direct delivery of siRNA to respiratory epithelia is potentially advantageous for pulmonary infections and for chronic diseases like cystic fibrosis, where airway epithelial cells are prominent sites of production and release of proinflammatory cytokines, including interleukin-8 (IL-8).¹-³ Topical delivery may avoid hepatic or renal clearance and nonspecific accumulation associated with systemic routes. However, due to its high molecular weight and polyanionic nature, siRNAs do not cross the epithelial cell membrane freely. Additionally, mucus presents an intra pulmonary physical barrier to overcome prior to cell entry. Thus, efficient delivery of siRNAs to the airways is challenging due to intracellular and extracellular barriers.

Nonviral siRNA delivery is an attractive approach for achieving gene silencing in airway epithelia. A number of studies report successful delivery of naked siRNA to airways, especially for counteracting viral infections.⁴-⁶ However, recent reports show that siRNAs delivered intranasally or intratracheally do not enter lung cells and do not elicit RNA interference (RNAi).⁷ Early reports of siRNA efficacy were likely confounded by the off-target immunostimulatory effects of siRNA constructs.⁸-¹¹ Here, we explored further the utility of nonviral based siRNAs in reducing target abundance in well-differentiated primary airway epithelial cells. We show that well-differentiated epithelia are almost completely refractory to entry of siRNA unless subjected to certain treatments.

Results

Dicer-substrate siRNAs (DsiRNA) delivery has no silencing effect in well-differentiated cultures

Our overall goal was to test the effectiveness of DsiRNAs in well-differentiated primary airway epithelial cells. Well-differentiated airway epithelia maintained at the air–liquid interface (ALI) mirror to a great extent the in vivo morphology of the airway and thus are an ideal system to test efficacy of inhibitory RNAs in the form of DsiRNAs and nonviral delivery reagents.¹² We tested various nonviral reagents or siRNA enhancements, including cationic polymers (PEI or TAT-PEI), modified
naked siRNA (Accell), a peptide reagent (PTD-DRBD or Transductin) and a lipid transfection reagent (RNAiMAX). No hypoxanthine–guanine phosphoribosyltransferase (HPRT) silencing was observed after apical transfection of HPRT DsiRNA into pig airway epithelia (PAE) with polymers, Accell or Transductin (Figure 1a). Nor was there a reduction of HPRT mRNA levels in human airway epithelia (HAE) following transfection of siRNA conjugated with RNAiMAX or Transductin (Supplementary Figure S1). However, the DsiRNAs are functional based on their utility to reduce target gene expression (>90%) in PKI cells, a pig kidney cell line grown on plastic (Supplementary Figure S2). Thus, well-differentiated airway epithelia are not amenable to DsiRNA transfection with a broad range of transfection reagents; consequently, RNAi against the targets is not achieved.

Well-differentiated cells show minimal uptake of DsiRNA

We next investigated whether failure of silencing is due to limited DsiRNA uptake. We labeled DsiRNA internally with Digoxigenin (DIG) nucleotides and transfected them into cells using Transductin. Subsequently, the cell layers were fixed and processed for immunodetection of DIG label using fluorescent labeled anti-DIG antibodies. Confocal imaging of the cells revealed little to no internalization of DsiRNA in both pig airway cells (Figure 1b,c) and human airway cells (Supplementary Figure S3a,b). The results suggest that the inability of the epithelia to silence gene expression is largely due to a failure of the DsiRNA to enter the cells.

KGF, EGTA, and LLnL treatment of cells prior to siRNA treatment have no effect on target silencing

The decreased susceptibility of well-differentiated cells to DsiRNA prompted us to investigate the effect of interventions known to influence cellular processes including proliferation, permeability, and processing that might influence siRNA transfection and silencing. Keratinocyte growth factor (KGF) is a member of the fibroblast growth factor family. We and others have shown that KGF stimulates proliferation of differentiated human tracheal and bronchial epithelia. Pretreatment of PAE with KGF before transfection of DsiRNAs with either RNAiMAX or Transductin did not improve gene silencing relative to controls (Supplementary Figure S4a). In addition, we also treated the cells with EGTA, a calcium chelator, which causes a reversible increase in paracellular permeability. Similar to results seen with KGF, DsiRNA transfection with either RNAiMAX or Transductin did not silence HPRT after treatment with EGTA (Supplementary Figure S4b). Lastly, we treated the cells with the proteasome inhibitor [N-acetyl-L-Leucyl-L-Leucyl-L-Leucyl-L-Norleucinal (LLnL)] before transfection. LLnL is a tripeptide proteasome inhibitor that enhances recombinant adeno-associated virus type 2 transduction from the apical surface of HAE by modulating the intracellular trafficking and processing of the virus. Treatment with LLnL had no effect on silencing of HPRT following transfection of specific DsiRNA with either RNAiMAX or Transductin (Supplementary Figure S4c). Thus, well-differentiated cells are refractory to siRNA transfection with physiological manipulations that induce proliferation, changes in paracellular permeability, or proteasome inhibition.

DsiRNA delivery effectively silences targets in poorly differentiated epithelia

Since well-differentiated cells exhibit significant extracellular barriers to siRNA entry, we hypothesized that poorly differentiated cells, which lack many of the barriers present in mature cells, might be conducive to siRNA entry and knockdown of the target gene. In contrast to well-differentiated cells, poorly differentiated epithelia lack ciliated and goblet cells and do not have a pseudostratified columnar architecture. On transfection of poorly differentiated PAE, also grown at the ALI on culture inserts (2-day-old post-seeding) with DsiRNA against HPRT, mRNA levels were suppressed with all transfection reagents used. HPRT mRNA levels were reduced by 40% with RNAiMAX, 60–70% with Accell, and ~50–60% with Transductin (Figure 2). Similar reductions were found on transfection with DsiRNA against pig IL-8 (Supplementary Figure S5a) or when poorly differentiated human airway cells were transfected with similarly formulated HPRT-targeting.
DsiRNAs (Supplementary Figure S5b). These results demonstrate that before differentiation airway epithelia are conducive to DsiRNA transfection and target knockdown.

**DsiRNAs enter poorly differentiated transfected cells**

We next examined whether DsiRNA internalization was enhanced in poorly differentiated epithelia. Two days after seeding, cells were examined for DsiRNA uptake. In contrast to results in well-differentiated epithelia (Figure 1b), abundant DsiRNA internalization was observed in poorly differentiated epithelia from both pig (Figure 2b,c) and human (Supplementary Figure S3c,d). These data indicate that DsiRNAs can silence targets in poorly differentiated airway epithelia by virtue of their ability to enter the cells.

Airway epithelia become progressively resistant to RNAi when grown at ALI

We next investigated how time in culture influenced siRNA entry and target knockdown. Primary airway epithelia, maintained at ALI, take a minimum of 2–4 weeks after seeding to fully differentiate into a pseudostratified columnar epithelium.17 Cells obtained from two pig donors were seeded and batches of cells from 2 to 5 days post-seeding were transfected with HPRT-targeting DsiRNAs complexed with Transductin. Reduction in HPRT mRNA by 20–45% was seen in 2–4-day-old cultures, but there was no silencing in 5-day cultures (Figure 3a). The cultures were also visualized for DsiRNA entry after DIG-DsiRNA transfection. The presence or absence of target knockdown correlated with DIG-DsiRNA internalization (Figure 3b–e). Cells transfected with DsiRNA against IL-8 also showed decreased IL-8 mRNA in 2-day-old, but not in 5-day-old cultures (Supplementary Figure S6a). Similarly, HAE showed no HPRT knockdown when DsiRNAs were applied after 5 days in culture (Supplementary Figure S6b). Thus, silencing of targets is restricted to a narrow time window post-seeding before cell differentiation reaches the point that the cells are refractory to RNAi approaches. Notably, cells undergo differentiation but not extensive cell division during this time frame.

**EGF pretreatment enhances siRNA entry and silencing**

We hypothesized that manipulation of the cells to enhance endocytosis might promote better cellular siRNA uptake and silencing of target genes. Epidermal growth factor (EGF) induces rapid actin filament assembly in the membrane

[Figure 2](#) DsiRNAs effectively silence targets in poorly differentiated PAE cultures. (a) Poorly differentiated cultures, 2-day post-seeding, were transfected with 250 nmol/l of DsiRNA against HPRT with the use of RNAiMAX, Transductin, or Accell siRNA at the indicated concentrations and then processed (RNA isolation, RT-qPCR) 24 hours later. Remaining mRNA levels were normalized to negative control (NC1). Mean levels (±SD) were calculated from three biological replicates (in triplicate) **P < 0.01, ***P < 0.001 (Student’s t-test). (b–c) Confocal images (x–z stacks) of epithelia 2 hours after transfection with DIG-HPRT DsiRNA complexed with (b) Transductin or (c) unformulated. Blue, labeled nuclei; green, DIG-labeled oligo.

[Figure 3](#) DsiRNA efficacy as a function of time in culture. (a) PAE cultures that were 2–5 days old were transfected with DsiRNA against HPRT (250 nmol/l) using Transductin. The cells were processed for RNA isolation and cDNA synthesis 24 hours later and then subjected to RT-qPCR for quantification of HPRT mRNA. HPRT levels were normalized to NC1 samples. Mean levels (±SD) were calculated from three biological replicates (in triplicate), **P < 0.01, ***P < 0.001 (Student’s t-test), (b–e) Confocal images (x–z stacks) of (b) 2, (c) 3, (d) 4, and (e) 5-day-old cells after transfection of each with DIG-HPRT DsiRNA and then staining 2 hours later for DIG. Red, nuclei; green, DIG-labeled oligo.
Cmap analysis links gene signatures of mucociliary differentiation to small molecules

We next took advantage of the Connectivity map (Cmap) to identify pathways that, when manipulated, could overcome the resistance of well-differentiated airway epithelia to RNAi. In brief, the Cmap provides users with a dataset of transcriptional changes that occur in a number of cell lines in response to a drug treatment. Investigators merge this dataset with expression data from a system of interest, in which gain-of-function and loss-of-function expression studies have been done.

Here, we used previously published data sets representing immature airway epithelia (which are receptive to siRNA delivery and activity) and well-differentiated airway epithelia (which are resistant to siRNA delivery and activity). The rationale is that gene expression changes which underlie the state of differentiation in some way contribute to the emerging resistance of these cells to RNAi. Drugs known to induce pathways active in the more de-differentiated physiological state may provide a “permissive environment,” and allow for siRNA activity even though the cells remain as a mature epithelium. To accomplish this, we used the data set previously reported by Ross et al., which identified genes involved in the differentiation of human bronchial epithelial cells grown at the ALI over 28 days. We found several small molecules which positively or negatively correlated to the reference signatures. Because we were interested in agents that might induce cell physiological changes which mimicked pathways active in poorly differentiated cells, we focused on drugs which correlated negatively with the reference signatures. Four agents were selected that ranked highly with a strong anti-correlation score (connectivity score of −1 or close to −1). These included LY294002, ergocalciferol, paclitaxel, and nifedipine. Two additional agents that were highly ranked in the results according to their $P$ values were also selected: naltrexone and fasudil. As a negative control in our experiments, we selected the drugs monensin and meclofenamic acid; each had strong positive correlation scores (connectivity score of +1 or close to +1).

We next treated primary airway epithelia with the selected small-molecule agents. Cells were treated for 6 hours, then transfected with DsiRNA–Transductin complex for 24 hours. When pig or human epithelia were transfected with the HPRT-targeting DsiRNA, we observed no reduction in mRNA levels in epithelia treated with ergocalciferol, paclitaxel, nifedipine, naltrexone, or fasudil (data not shown). In contrast, following
with increasing doses of wortmannin and triciribine before DsiRNA transfection resulted in dose-dependent improvements in RNAi efficacy (Figure 5d). Treatment of cells with 10 and 40 nmol/l of wortmannin decreased HPRT levels by 20 and 30%, respectively, compared to controls. Treatment with 20 and 60 µmol/l triciribine resulted in 20 and 35% decrease, respectively in HPRT mRNA levels compared to controls (Figure 5d). These findings suggest that the improved silencing of the siRNA target is a consequence of cellular changes that occur in response to PI3K pathway inhibitors. Finally, we also tested the effects of LY294002 treatment on two other DsiRNA gene targets, cystic fibrosis transmembrane conductance regulator (CFTR) and SIN3A. Pretreatment of human airway cells with 10 µmol/l of LY294002 before transfection with these specific DsiRNAs resulted in 35 and 25% reduction in CFTR and SIN3A mRNA levels, respectively, when compared to control (Supplementary Figure S7a,b).

LY294002 pretreatment decreases CFTR protein levels in epithelia transfected with CFTR DsiRNA

We next tested whether this level of gene silencing was sufficient to reduce the expression of a protein important in lung
cell physiology, the CFTR protein. CFTR encodes an anion channel in airway epithelia and other cells.31,32 Loss of CFTR function, caused by mutations in the CFTR gene causes cystic fibrosis, an important chronic disease characterized by progressive pulmonary infection and inflammation.33 Treatment of HAE with LY294002 before transfection of cells with CFTR-targeting DsiRNA formulated in Transductin resulted in 35% reduction in CFTR mRNA levels as shown in Supplementary Figure S7b. Analysis of CFTR protein by western blot showed decreased protein levels in transfected cells treated with LY294002 compared to controls (Figure 6). Both band B and mature, fully glycosylated CFTR band C were significantly decreased in drug-treated cells. The decrease in protein levels was observed in cells from three separate donors tested (data not shown). Thus, well-differentiated airway epithelia, normally refractory to siRNA delivery, are amenable if pretreated with LY294002, allowing for silencing of target mRNA and protein levels.

**Discussion**

siRNA-mediated silencing of gene expression holds great promise for disease treatment. While there has been much progress in siRNA design to avoid immunogenicity, toxicity, and off-target effects, one of the remaining hurdles is delivery.34 Unlike many organs, the lung permits direct access of delivery materials to the target cells via topical administration through intranasal, intratracheal, and aerosol routes. Thus, many lung diseases which have epithelial cell pathologies, namely cystic fibrosis, chronic obstructive pulmonary disease, pulmonary fibrosis, and respiratory viral diseases, are ideal targets for siRNA treatment. In this study, we tested DsiRNA-nonviral reagent combinations for effective silencing of gene targets by applying various formulations to the apical surface of primary pig or HAE cells. We used DsiRNAs previously shown to evoke potent RNAi.35

Primary airway epithelia from either trachea or bronchi and grown in an ALI culture become well-differentiated over time, form a pseudostratified columnar epithelium with tight junctions, develop cilia, and produce mucin.12,17 Previous studies showed a strong overall correlation in gene expression between cells grown at ALI and cells obtained directly from in vivo bronchial brushings,12 supporting this culture system as an excellent model for studies of lung epithelium biology. One prior report noted that well-differentiated epithelia transfected apically with siRNA formulated with Oligofectamine or jetSI did not reduce target gene expression.36 And, although siRNA entry was described as limiting, no data were presented. Here, we found that none of our DsiRNA formulations were successful in gene silencing to well-differentiated epithelia, irrespective of the dose or time of transfection, and this correlated strongly with limited oligonucleotide entry.

Thus, successful delivery of DsiRNA to the airways depends on the capacity of the formulations to overcome a variety of barriers. One of these is the mucus biopolymer network, which causes steric obstruction and thereby hinders diffusion of lipid or polymer nanoparticles.37,38 In addition, macromolecules such as antibodies may interact with nonviral nanoparticles and cause entrapment, aggregation or release of the RNA. However, in our experiments, repeated washings of the apical surface with phosphate-buffered saline, which removes most of the mucus, did not improve RNAi efficacy.

Several different formulations have been investigated for in vivo siRNA delivery to the lungs. Therapeutic efficacies were reported from intranasal or intratracheal administration of naked siRNA, including reduction in the titers of airway-tropic viruses, or reducing transcripts pivotal in airway disease.4–6,9,10,39–43 However, misinterpretation may have arisen from the use of control sequences that were less immunostimulatory than the siRNA sequences targeting the gene of interest.5–11 Additional work indicated that direct intratracheal administration of naked siRNA into mouse lung did not achieve target knockdown.7 Instead, as early as 15 minutes after administration renal excretion was observed, indicating rapid absorption across the respiratory epithelium without
We found that target gene silencing was effective in poorly differentiated pig and HAE as a function of improved entry. Primary cultures of different ages are distinct in their makeup of cell types, average apical membrane surface area, and overall architecture. Also, tight junctions that regulate paracellular passage of molecules are present in epithelia within a day of seeding. This suggests that poor DsiRNA entry is not related to the formation of tight junctions. This interpretation was supported by studies with EGTA of well-differentiated epithelia. Previous studies showed that EGTA treatment of airway epithelia in vitro and in vivo enhanced gene transfer with retroviruses and adenovirus by providing access to the basolateral surface. EGTA treatments did not enhance target silencing with DsiRNA formulations, however.

We also considered that actively proliferating cells would enhance entry, since cells early post-seeding are still dividing (albeit slowly) and they were more susceptible to RNAi. However, inducing well-differentiated epithelia to divide and proliferate did not improve gene silencing. In spite of this result, we cannot conclude that cell division has no impact on entry. Another consideration is that the cellular makeup within the epithelia changes as a function of differentiation.

The failure of siRNA to enter cells efficiently after just 5 days post-seeding implies that barrier properties arise before complete differentiation of the epithelia. This suggests that physiological and structural differences associated with cell growth and division may have a bearing on siRNA entry. Cell-specific differences in transfection efficiency have been attributed to effects of not only cell cycle and cell division frequency, but also to endocytic capacity. It has been established that endocytosis acts as a major path of entry for many polyplexes-containing nucleic acid. Lipoplexes or PEI polyplexes are internalized by various endocytic pathways, including macropinocytosis, clathrin-mediated endocytosis, and nonclathrin-mediated mechanisms with entry via caveolae. Two studies show that the protein transduction domain Tat, which is the cell-penetrating domain of the peptide agent Transductin, enters cells by macropinocytosis. For these reasons, we tested EGF. EGF acts through a tyrosine kinase receptor (EGFR) to rapidly stimulate actin reorganization at the cell membrane resulting in membrane ruffling and macropinocytosis. EGFR can be activated in the airways and has been implicated in epithelial cell repair and mucin production. Additionally, matrix metalloproteinase-9, which is abundant in various chronic airway disorders and involved in lung remodeling, induces release of membrane bound EGF-like ligands at the apical surface that then subsequently bind to EGFR. We hypothesized that treatment of EGF could enhance internalization and endocytosis of the siRNA cargo, especially those formulated with Transductin since its entry is shown to occur by macropinocytosis. EGF treatment of human primary airway epithelia prior to transfection of DsiRNA with Transductin increased knockdown from 0 to 15–20%. Consistent with this, confocal imaging revealed increased internalization of the DIG-labeled DsiRNA. It will be interesting to test how further modulation of this pathway may impact siRNA delivery to airway.

A resource that provided insight regarding the differences between poorly- and well-differentiated airway epithelia was the molecular atlas of gene expression changes during mucociliary differentiation. Many of the functional categories of the genes correlated well with the transformation of these cells into a pseudostratified ciliated epithelium that shares many properties of the in vivo airway epithelium. We used three gene signatures (0 versus 4 day; 0 versus 8 day; 4 versus 8 day) from this dataset to query the Cmap database in an attempt to link genes associated with differentiation to candidate small molecules. Treatment with five of the six compounds selected did not enhance DsiRNA delivery or activity in well-differentiated airway epithelia. One of the small molecules, LY294002, a PI3K inhibitor, improved knockdown in pig airway and was therefore also tested in HAE. There its impact to the formulation was more robust. Of note, the Cmap data was generated in human cell lines and thus the resource may be less useful for predicting small molecules that impact gene expression changes in other species.

We also show that manipulation of well-differentiated epithelia with LY294002 facilitates knockdown of disparate DsiRNA targets (SIN3A and CFTR). Silencing of CFTR mRNA by this approach also reduced levels of the CFTR protein. Thus, the drugs we have identified in this study (the PI3K pathway inhibitors) provide a permissive environment for siRNA activity to function in a system that has proved previously to be extremely difficult to access for nucleic acid delivery by nonviral methods.

PI3Ks have key regulatory roles in many cellular processes, including cell survival, proliferation, and differentiation. PI3Ks are downstream effectors of receptor tyrosine kinases and G protein-coupled receptors and transduce signals from growth factors and cytokines. These signals activate serine-threonine protein kinase AKT and other downstream effector pathways. Many components of the pathway are mutated in human cancers. LY294002 was the first synthetic drug-like small molecule inhibitor capable of reversibly targeting PI3K family members in the micromolar range. The observed enhancement of LY294002 on airway DsiRNA silencing would suggest that treatment with other PI3K pathway inhibitors might have similar effects. We tested two compounds outside of the Cmap database, wortmannin (a PI3K inhibitor) and triciribine (an Akt inhibitor) and observed a dose-dependent silencing effect in HAE with both compounds, suggesting that PI3K pathway inhibition is playing a role. All three drugs inhibit tumorigenesis. However, how these molecules increase siRNA uptake and silencing remains obscure.

In conclusion, we discovered that well-differentiated airway epithelia are refractory to treatment with commonly used siRNA and DsiRNA formulations. This barrier occurs at least in part at the level of cargo entry. Importantly, manipulation of epithelia can induce effective DsiRNA uptake. Further refinements and studies of the mechanism behind these barrier changes may aid in developing additional successful strategies for gene silencing in well-differentiated epithelia, and translating RNAi to in vivo applications.
Materials and methods

Culture of primary epithelial cells and cell lines. PAE cells were obtained from trachea of lungs removed from euthanized pigs. The studies were approved by the institutional review board at the University of Iowa. HAE cells were obtained from trachea and bronchi of lungs removed for organ donation from non-CF individuals. Cells were isolated by enzyme digestion as previously described.35 Following enzymatic dispersion, cells were seeded at a density of $5 \times 10^5$ cells/cm² onto collagen-coated, 0.6-cm² semipermeable membrane filters (Millipore polycarbonate filters; Millipore, Bedford, MA). The cells were maintained at $37^\circ$C in a humidified atmosphere of 5% CO₂ air. Twenty-four hours after plating, the apical media was removed and the cells were maintained at an ALI to allow differentiation of the epithelium. The culture medium consisted of 1:1 ratio mix of Dulbecco’s modified Eagle’s medium/Ham’s F12, 5% Ultroser G (Biosepra SA, Cedex, France), 100 U/ml penicillin, 100 µg/ml streptomycin, 1% nonessential amino acids, and 0.12 U/ml insulin. Studies were performed on well-differentiated epithelia.4–6 weeks after initiation of the ALI cultures conditions. Pig kidney cells (PK1) were cultured in Medium 199 (Life Technologies, Carlsbad, CA) supplemented with 5% fetal bovine serum.

DsiRNA oligonucleotides. The 27-mer DsiRNAs were designed using algorithms developed by Integrated DNA Technologies (IDT, Coralville, IA) and synthesized and high-performance liquid chromatography purified by IDT. The protocol for DsiRNA design and manufacture has been described in detail.35,48 The DIG-labeled DsiRNA was also designed and synthesized by IDT. The DIG label was internally coupled to an amino-dT base in a 2-O’ methyl modified pig-specific DsiRNA against HPRT. The DsiRNAs used in this study are listed in Supplemental Table S1.

RNAi reagents. Lipofectamine RNAiMAX was purchased from Invitrogen (Life Technologies). The cationic polymers, PEI, and TAT-PEI were synthesized and conjugated with DsiRNA into appropriate NP concentrations. Accell siRNA (Thermo Scientific, Lafayette, CO), which are chemically modified to increase stability and enhance uptake by cells, were custom synthesized as 21-nucleotide siRNA with a 5′ base in a 2-O′ methyl modified pig-specific DsiRNA against HPRT. The DsiRNAs used in this study are listed in Supplemental Table S1.

Transfection. For airway epithelia that were either well or poorly differentiated, transfection was performed mostly according to the manufacturer’s protocol. In all cases, the apical surface of epithelia was washed twice with phosphate-buffered saline before adding the transfection mixture to the apical surface. The mixture was then left on the cells for 24 hours for all transfection reagents. For PK1 cells, reverse transfection was performed in a 48-well plate by first adding the DsiRNA-RNAiMAX reagent mixture onto the plate and then adding the dispersed cells (40,000 cells), allowing the cells to adhere and establish growth for 24 hours. Before some transfections, cells were pretreated with various chemicals: KGF (Prospec, East Brunswick, NJ) 50 ng/ml added both apically and basolaterally for 24 hours; EGTa 6 mmol/l added apically for 30 minutes LLnL (ICN Biochemicals, Costa Mesa, CA) 40 µmol/l added both apically and basolaterally for 24 hours; human EGF (Sigma-Aldrich, St Louis, MO) 100 µg/ml, added apically for 15 minutes. For all experiments, a minimum of three biological replicates, in triplicate, were done.

RNA isolation and quantitative real-time PCR. Total RNA was isolated using SV96 RNA isolation kit (Promega, Madison, WI), according to manufacturer’s protocol. Total RNA (250 ng) were reverse transcribed using oligo (dT) (Roche Biochemicals, Indianapolis, IN) and random hexamers (Life Technologies) and Superscript II (Life Technologies) according to manufacturer’s instructions. One-fifteenth of the cDNA was then amplified and analyzed by Taqman assay in the 7900 Real-Time PCR System (Applied Biosystems, Foster city, CA) using synthesized primer-probe pairs (IDT), reaction buffer and Immolase DNA polymerase (Bioline, Taunton, MA). All the primers and probes used in the study are listed in Supplemental Table S2. The reaction mix was contained in a total volume of 10 µl and the reaction condition was an initial cycle of 95 °C for 10 minutes, then 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minutes. All data were normalized to the internal standard, RPL4 mRNA for pig airway samples and SFRS9 mRNA for human airway samples. Absolute quantification of an mRNA target sequence within an unknown sample was determined by reference to a standard curve. The standard curve was based on the real-time PCR amplification of standard amounts of the specific gene in a control NC1 treatment cDNA. PCR efficiency for all reactions was within the acceptable margin of 90–110%. All results of the samples were presented as remaining target mRNA level in comparison to the mRNA level in the control samples (NC1), which was normalized to 100%.

Confocal imaging. The primary HAE grown at ALI were transfected with the DIG-labeled DsiRNA at a concentration of 250 nmol/l after complexing with Transductin. The transfection mixture was left on the apical surface for 2 hours. At the end of this period, the cells were fixed in 2% formaldehyde, permeabilized in 0.2% Triton-x-100 and blocked in 1% bovine serum albumin for 1 hour. The cells were then stained with mouse antibody to DIG (Roche Biochemicals) for 1 hour followed by Alexa 488-labeled goat anti-mouse secondary antibody for 1 hour and then Alexa 488-labeled rabbit anti-mouse tertiary antibody (Life Technologies). In some cases Alexa 564 goat anti-mouse secondary antibody was used. The cells were finally stained with nuclear stain, ToPro 3 for 10 minutes. The filter, containing the cells, was removed from the culture insert by cutting the edges with razor blade, mounted with Vectashield (Vector Laboratories, Burlingame, CA). The cells were visualized by confocal microscopy (Bio-Rad Radiance 2100MP Multiphoton/Confocal Microscope). For each experiment, epithelia from a minimum of three donors were analyzed, with biological replicates done in duplicate.

SDS-PAGE and immunoblotting. Human primary culture was washed with phosphate-buffered saline and lysed in freshly prepared lysis buffer (1% Triton, 25 mmol/l Tris pH 7.4, 150 mmol/l NaCl, protease inhibitors (Complete; mini, EDTA-free; Roche Biochemicals) for 30 minutes at 4 °C. The lysates were centrifuged at 14,000 rpm for 20 minutes at 4 °C, and the supernatant
quantified by BCA Protein Assay kit (Pierce, Rockford, IL). Fifty nanogram of protein per lane was separated on a 7% SDS-PAGE gel for western blot analysis. CFTFR antibody (R-769) was procured from Cystic Fibrosis Foundation and used at a dilution of 1:2000; α-tubulin antibody was purchased from Sigma (1:10,000 dilution). Protein abundance was quantified by densitometry using an AlphalnnotechFluorchem Imager (Alpha Innotech, Santa Clara, CA). Band B and C of CFTFR protein were quantified separately. Polyvinylidene difluoride membrane was first probed with the R-769 anti-CFTFR antibody, imaged and then stripped and reprobed as follows. The membrane was stripped with Restore Western Blot Stripping Buffer (Thermo Scientific) for 15 minutes, washed in Tris-buffered saline-Tween (TBS-T) and blocked in 5% bovine serum albumin (Pierce) for 1 hour. The membrane was then reprobed for α-tubulin.

Cmap analysis. The Cmap is a public database of human cell line gene expression data sets representing responses to drug treatments that can be queried using input gene expression signatures to identify small molecules that share similar gene expression patterns.23 We used input query signatures derived from published microarray data26 of gene expression changes associated with in vitro mucociliary differentiation of human bronchial epithelial cells. Specifically, genes with more than threefold expression change from 0 to 4 days culture or 0 to 8 days were used as input signatures (probe ID defined by the Affymetrix GeneChip Human Genome U133A array). Each reference signature in the database was compared with the input signature and given a score termed the “connectivity score” based on the extent of similarity between the two. Scores ranged from +1 (+ correlation), 0 (no correlation), and −1 (reverse correlation). We selected candidate agents with connectivity scores approximating −1.

Small molecules. Wortmannin was purchased from Sigma as a ready to use solution. LY294002, triciribine hydrate, and meclofenamic acid were purchased from Sigma and were dissolved in DMSO. Monensin was purchased from Sigma and was dissolved in water. For experiments with drugs, the epithelia were pretreated with drugs for 6 hours at concentrations used in the Cmap study or available in the published literature. After drug treatment, the epithelia were transfected with DsiRNA as described before.

Supplementary Material

Figure S1. DsiRNA delivery has no silencing effect in well-differentiated HAE cultures.

Figure S2. Dicer-substrate DsiRNA silence gene expression in PK1 cells.

Figure S3. Confocal imaging of human airway epithelial cells transfected with Dig-HPRT DsiRNA.

Figure S4. DsiRNA delivery has no silencing effect in well-differentiated PAE cultures treated with EGTA, LLLnL, or KGF.

Figure S5. DsiRNAs effectively silence targets in poorly differentiated epithelial cultures.

Figure S6. Effective silencing of targets in 2-day-old cultures.

Figure S7. Small molecule treatment of epithelia prior to DsiRNA delivery improves target silencing.

Table S1. DsiRNAs

Table S2. qPCR primers and probes.

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Supplementary Information accompanies this paper on the Molecular Therapy–Nucleic Acids website (http://www.nature.com/mtna)