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RESEARCH PAPER

Histological Quantification of Gene Silencing by Intratracheal Administration of Dry Powdered Small-Interfering RNA/Chitosan Complexes in the Murine Lung

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ABSTRACT

Purpose The use of small-interfering RNA (siRNA) as an inhalation therapy has recently received much attention. Some reports have confirmed the suppression of gene expression in whole lungs following intratracheal administration of dry powdered siRNA; however, the anatomical location in the lung where gene silencing occurs has not been precisely identified. Here, we aimed to histologically evaluate gene silencing efficacy in murine lungs by intratracheal administration of an siRNA/chitosan complex as a dry powder.

Methods Enhanced green fluorescence protein (EGFP)-specific siRNA (EGFP-siRNA)/chitosan powder was prepared and administered intratracheally to EGFP transgenic mice or mice carrying metastatic lung tumors consisting of Lewis lung carcinoma (LLC) cells stably expressing EGFP (EGFP-LLCs). Thereafter, green fluorescence intensities were quantified in the airways, parenchyma, and lung tumors.

Results Intratracheal administration of the EGFP-siRNA/chitosan powder suppressed EGFP expression in the bronchi, bronchioles, and alveolar walls of EGFP transgenic mice. Additionally, EGFP-siRNA/chitosan effectively silenced EGFP expression in lung tumors consisting of EGFP-LLC cells.

Conclusions Pulmonary administration of siRNA/chitosan powder suppressed gene expression throughout the lung and in lung tumors. Therefore, this may become a powerful strategy to target genes expressed in a wide range of respiratory diseases involving the airways, parenchyma, and lung tumors.

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KEY WORDS airway epithelium · alveolar wall · dry powdered siRNA/chitosan complex · intratracheal administration · metastatic lung tumor

ABBREVIATIONS

LLC	Lewis lung carcinoma
EGFP-LLC	Lewis lung carcinoma cells expressing EGFP

INTRODUCTION

RNA interference (RNAi), which was first discovered in 1998 (1), is a mechanism of silencing gene expression after the gene has been transcribed. In this process, a short RNA duplex called small-interfering RNA (siRNA) interacts selectively with a single target sequence within mRNA, providing sequence-specific mRNA degradation and inhibition of protein production. RNAi has become a powerful tool to investigate gene function in mammalian cells, and, in recent years, siRNA has been employed as a potential therapeutic agent to directly block the expression of disease-associated genes. The lung is an attractive target organ for siRNA delivery because of its accessibility from the airways. In fact, direct intrapulmonary delivery of siRNA has been carried out for efficient gene silencing in the lungs of small animals. However, for clinical applications, the development of inhalable siRNA formulations is needed.

At present, three types of inhalation systems are clinically used: pressurized metered dose inhalers (MDIs), nebulizers, and dry powder inhalers (DPIs). MDIs are the most commonly used inhalers; however, they require propellants, such as chlorofluorocarbons and hydrofluoroalkanes. Chorofluorocarbons are known to damage the ozone layer of the earth, and hydrofluorocarbons have a relatively high global warming potential compared to carbon dioxide (2,3). Nebulizers are also widely used in clinical practice; however,

the nebulization process is associated with loss of the formulation and is therefore not as efficient. In addition, nucleic acids are most likely unstable in liquid formulation (2). Therefore, the use of dry powder of siRNAs as an inhalant has received much attention because this method does not require propellants and is compatible with the dry powder formulation of nucleic acids (4–6).

For the preparation of inhalable dry powder, the supercritical carbon dioxide (CO_2) technique may be useful as this technique has been used in the production of powders of several proteins, such as insulin, lysosome, and catalase, without losses of activity (6–8). In a previous study, we used mannitol and low-molecular-weight chitosan as a nonviral carrier of nucleic acid and succeeded in preparing the dry powder of plasmid DNA (3,9) and siRNA (10) with this technique. Moreover, in another previous study (10), intratracheal administration of this siRNA/chitosan powder containing siRNA against luciferase was shown to effectively reduce the gross luminescence intensity in mouse lungs bearing metastatic tumors consisting of cells expressing firefly luciferase. This result indicated that intratracheal administration of the siRNA/chitosan powder is effective at silencing gene expression in the lung; however, the anatomical location in the lung where gene silencing occurs has not yet been determined.

Therefore, in this study, we sought to histologically evaluate the gene suppression efficacy of siRNA in murine lungs following intratracheal administration of an siRNA/chitosan complex as a dry powder. To this end, enhanced green fluorescence protein (EGFP)-specific siRNA (EGFP-siRNA)/chitosan powder was prepared and was administered intratracheally to EGFP transgenic mice or mice carrying metastatic lung tumors consisting of Lewis lung carcinoma (LLC) cells stably expressing EGFP (EGFP-LLCs). Thereafter, green fluorescence intensities were histologically quantified in the airways, parenchyma, and lung tumors.

MATERIALS AND METHODS

Mice and Materials

Eight- to 10-week-old female C57BL/6 mice and C57BL/6-Tg (CAG-EGFP) mice (EGFP transgenic mice) were purchased from Japan SLC Inc. (Hamamatsu, Japan). EGFP-siRNA (Silencer GFP siRNA) was purchased from Life Technologies (Carlsbad, CA, USA). Noncoding siRNA (nontargeting siRNA) was obtained from Bonac (Kurume, Japan). Water-soluble chitosan (nominal molecular weight [Mw] = 2000–5000 Da; Wako Pure Chemical Industries Ltd., Japan) and mannitol (Wako Pure Chemical Industries Ltd.) were used as a nonviral vector and an excipient, respectively. Pentobarbital was purchased from Kyoritsu Seiyaku Corporation (Tokyo, Japan).

Preparation of siRNA/Chitosan Powder Using the Supercritical CO_2 Technique

Dry powder of siRNA/chitosan was prepared as previously described (10). In brief, 50 mg of materials (composition ratio: siRNA 2%, chitosan 10%, mannitol 88%) was dissolved in 1 mL of water. The solution was injected into the water flow through a manual injector. Flow rates of CO_2, ethanol, and water were set to 14 mL/min, 0.665 mL/min, and 0.035 mL/min, respectively. The three solvents were mixed in a compressed column (35EC, 25 MPa) to precipitate the components of samples. At 90 min after sample injection, the flows of water and ethanol were stopped, while that of CO_2 was continued for an additional 60 min to completely remove the water and ethanol in the column. Following the release of pressure, the dry powder was collected from the column. The powder was ground for 5 min manually using a pestle and mortar to improve its dispersibility. This powdering process was required to prepare siRNA/chitosan powders with good uniformity. In a previous study (10), we confirmed that the powdering process and dry grinding process had no effect on the particle size and Zeta potential of the siRNA/chitosan complex.

Fluorescence Imaging Experiment

Fluorescence microscopic studies were performed using a fluorescence microscope (BIOREVO BZ-9000; Keyence, Osaka, Japan) with an excitation filter (470/40 nm) for EGFP. We employed fixed settings for acquisition and processing of fluorescence images in each experiment in this study. The fluorescence intensity was measured, and the area of the region of interest (ROI) was calculated using an image-analyzing system (BZ-H2A/BZ-H1M/BZ-H1C, Keyence) equipped with the fluorescence microscope. As a quantification of fluorescence level, fluorescence density, defined as the sum of fluorescence intensity in the ROI divided by the sum of the area of the ROI, was utilized.

Intratracheal Administration of EGFP-siRNA/Chitosan Powder in Mice

Animals were maintained according to guidelines for the ethical use of animals in research at Hiroshima University. All animal experiments were approved by the Institutional Animal Care and Use Committee of Hiroshima University and were carried out in accordance with Hiroshima University Animal Experimental Regulations.

Intratracheal administration of siRNA/chitosan powder was conducted as follows:

1) A disposable yellow tip was connected to a 22-gauge catheter (Supercath 5, Medikit, Tokyo, Japan), and 1.5 mg of

siRNA/chitosan powder was placed into the yellow tip (Fig. 1a).

2) A 3-way stopcock at the closed position linked to a 1-mL syringe containing 0.3 mL of air was connected to the disposable yellow tip attached to the 22-gauge catheter (Fig. 1B-1).
3) Mice were anesthetized by intraperitoneal injection of pentobarbital and a board was secured on their backs by holding their upper incisor teeth. The tongue was extended using tweezers, and the mice were orally intubated with the 22-gauge catheter connecting to the disposable yellow tip, 3-way stopcock, and 1-mL syringe.
4) As shown in Fig. 1B-2 and B-3, the air in the syringe was compressed by pushing the plunger, and the dry powder in the yellow tip was dispersed into the trachea by turning the 3-way stopcock to the open position.

We carried out a multiple dosing regimen using intratracheal administration of the powdered siRNA in order to achieve a broader distribution of siRNA in the mouse lung. This approach has been reported in several other previous studies of multiple dosing regimens for pulmonary delivery of siRNA (10,11,13,14,23).

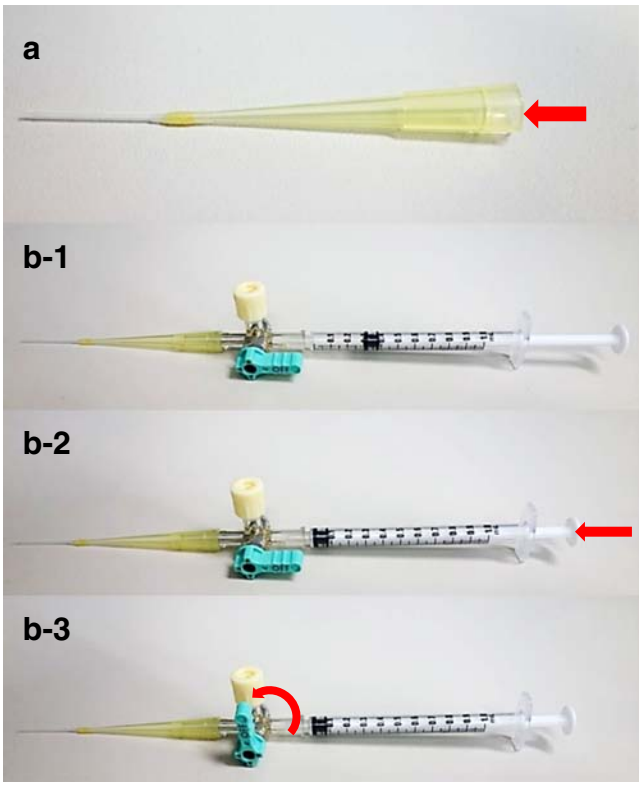


Fig. 1 Preparation and handling of the device to intratracheally administer siRNA/chitosan powder. (**a**) A disposable yellow tip was connected to a 22-gauge catheter, and 1.5 mg of siRNA/chitosan powder was placed into the yellow tip as indicted by the *arrow*. (*B-1*) A 3-way stopcock at the closed position linked to a 1-mL syringe containing 0.3 mL of air was connected to the disposable yellow tip attached to the 22-gauge catheter. (*B-2*) The air in the syringe was compressed by pushing the plunger as indicated by the *arrow*. (*B-3*) Dry powder in the yellow tip was dispersed by turning the 3-way stopcock to the open position as indicated by the *curved arrow*.

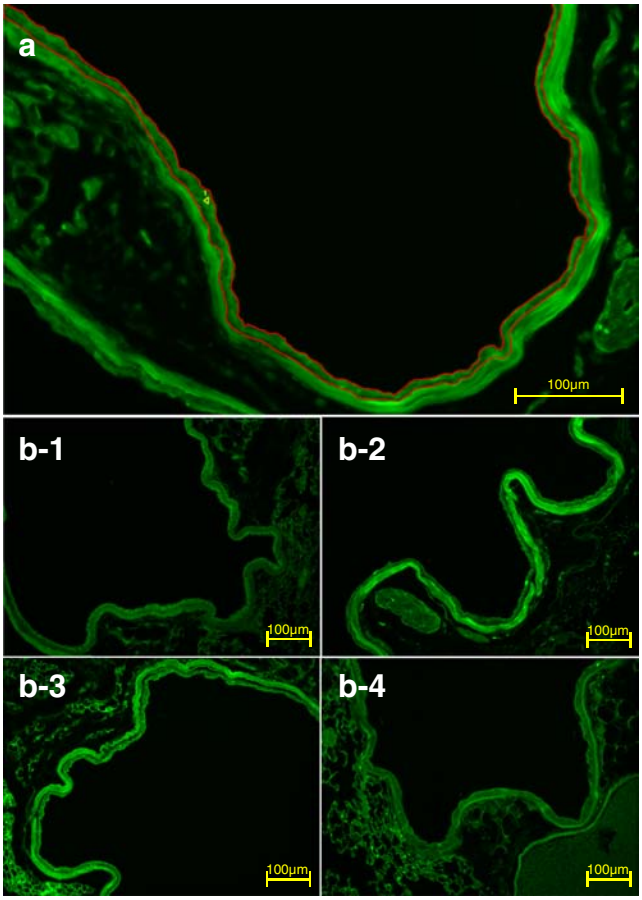
Quantification of EGFP Fluorescence in the Airways and Alveolar Walls of Mice

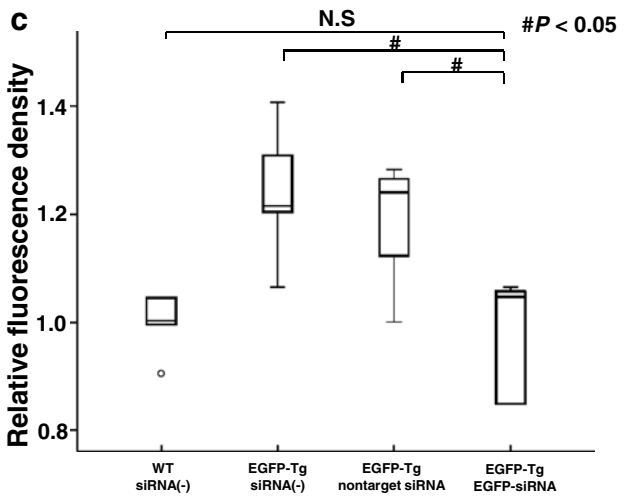
Mice were sacrificed with an overdose of pentobarbital, and lungs were immediately harvested, fixed with 4% paraformaldehyde in phosphate-buffered solution, and then embedded in paraffin. The whole lungs were sectioned in 5-μm-thick slices, and every 300th section was sampled. A total of 8 lung sections from each mouse were used for the next sampling step. Selected lung sections were deparaffinized and mounted on slides with Prolong Gold anti-fade reagents (Invitrogen, Carlsbad, CA, USA). These lung sections were viewed at a magnification of 40× with a fluorescence microscope. All the regions including the airways were enclosed within a fixed size of rectangular frame at a magnification of 200×. Every sixth rectangular frame view from each section was sampled for the subsequent quantification steps. The airways observed in the rectangular frame were categorized as either the bronchus or the bronchiole based on the histological morphology of the epithelium, and the epithelial regions of the bronchus and bronchiole were traced as shown in Figs. 2a and 3a, respectively. The fluorescence density was calculated for tissues within each category.

For the quantification of fluorescence levels in the alveolar region, every 600th 5-μm-thick section of whole lung was selected, and a total of 4 lung sections were sampled from each mouse. These sampled lung sections were deparaffinized and mounted on slides with Prolong Gold anti-fade reagents, blinded, and randomized among all the lung sections selected for the quantification of fluorescence level in the alveolar region. Each lung section was viewed at a magnification of 200× with a fluorescence microscope, and 4 circular frames including only alveolar compartments were randomly sampled. In each circular frame, regions whose fluorescence intensity exceeded a fixed level were highlighted in white to exclude alveolar air space and identify alveolar walls (Fig. 4a). Therefore, only alveolar walls were displayed in white in these circular frames, and the fluorescence density was calculated in these white highlighted regions. In this experiment, the threshold gradation was set at 40 in 256 gradations to identify alveolar walls in 8-bit (256 gradations) fluorescence images.

Establishment of Lung Metastatic Tumors Consisting of LLC Cells Stably Expressing EGFP and Quantification of EGFP Fluorescence in Metastatic Tumors

LLC cells were obtained from ATCC (Manassas, VA, USA). LLC cells stably expressing EGFP (EGFP-LLC) were established using the RetroMax System (IMGENEX Corporation, San Diego, CA, USA), cloned by limiting





◀ **Fig. 2** (**a** and *B-1* to *B-4*) Representative fluorescence images highlighting bronchi and (**c**) comparison of fluorescence density in the epithelium of the bronchus. (**a**) Representative image of traced epithelium in the bronchus. (*B-1*) Bronchus of a wild-type mouse without siRNA treatment. (*B-2*) Bronchus of an EGFP transgenic mouse without siRNA treatment. (*B-3*) Bronchus of an EGFP transgenic mouse treated with nontargeting siRNA. (*B-4*) Bronchus of an EGFP transgenic mouse treated with EGFP-siRNA. (**c**) The fluorescence density in the epithelium of the bronchus was quantified as described in the Materials and Methods. *WT siRNA (-)* wild-type mice without siRNA treatment, *EGFP-Tg siRNA (-)* EGFP transgenic mice without siRNA treatment, *EGFP-Tg nontarget siRNA* EGFP transgenic mice treated with nontargeting siRNA, *EGFP-Tg EGFP-siRNA* EGFP transgenic mice treated with EGFP-siRNA. The fluorescence density of the epithelium of the bronchus in the EGFP-Tg EGFP-siRNA group was significantly suppressed. $^{\#}P < 0.05$ *vs.* the EGFP-Tg siRNA (-) group and the EGFP-Tg nontarget siRNA group.

dilution, and cultured in RPMI1640 medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. Next, 1.0×10^6 EGFP-LLC cells in exponential growth phase were suspended in 0.1 mL of PBS and then injected intravenously into C57BL/6 mice through the tail vein. Mice were sacrificed 14 days after the injection of EGFP-LLC cells. After perfusion fixation with 4% paraformaldehyde in phosphate-buffered solution, the lungs were removed, embedded in OCT compound (Sakura Finetek, Tokyo, Japan), and snap-frozen in liquid nitrogen. Next, 20-μm-thick slices were sectioned from the frozen lungs at −25°C and mounted on slides with Prolong Gold anti-fade reagents. From each mouse, 10 sections were randomly selected and viewed with a fluorescence microscope at a magnification of 20×. In each section, regions whose fluorescence intensity exceeded a fixed level were highlighted in white, as shown in Fig. 5b, and identified as tumors consisting of EGFP-LLC cells. The fluorescence densities in such tumor regions were calculated, and the values for 10 lung sections were totaled for each mouse. In this experiment, the threshold gradation was set at 40 in 256 gradations to identify EGFP-expressing tumor regions in 8-bit (256 gradations) fluorescence images.

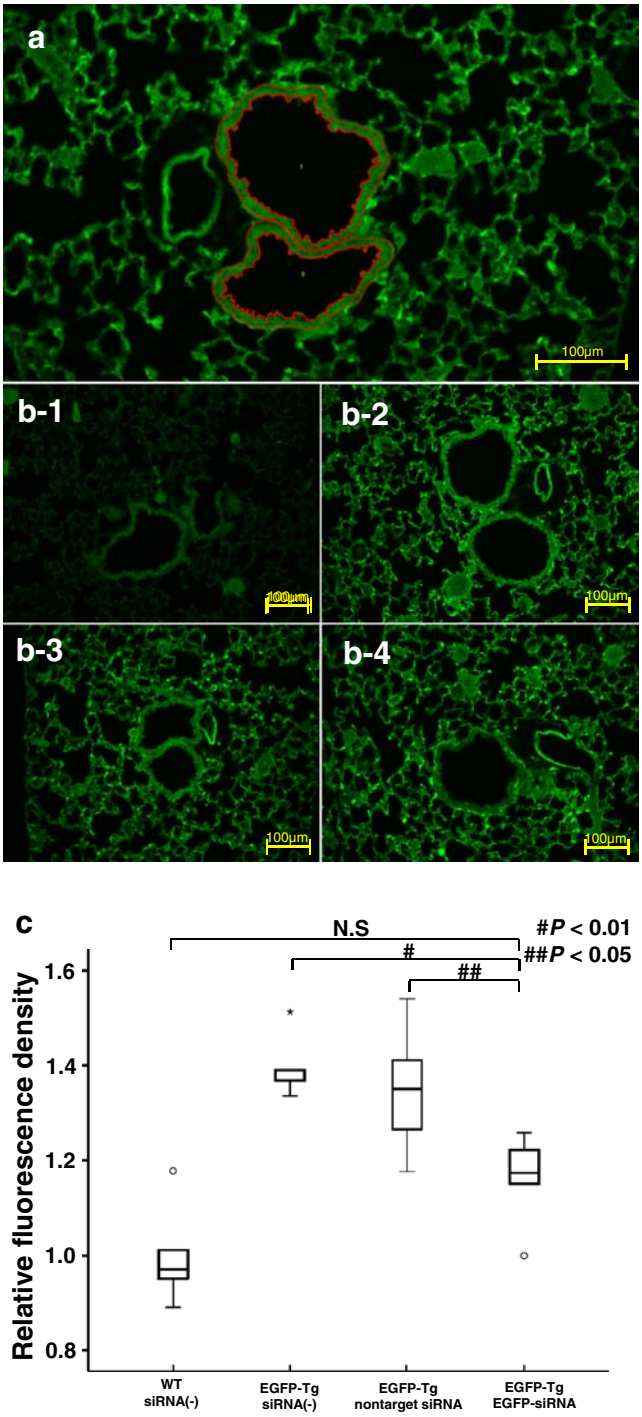
Statistical Data Analysis

Comparisons between each group of mice were made using the Mann–Whitney *U*-test. A *P* value of less than 0.05 was considered to be statistically significant. All analyses were performed using SPSS for Windows, version 19.0 (SPSS, Chicago, IL, USA).

RESULTS

Suppression of EGFP Fluorescence in the Respiratory Epithelium and Alveolar Walls of EGFP Transgenic Mice by Intratracheal Administration of EGFP-siRNA/Chitosan Powder

To determine whether intratracheal administration of siRNA/chitosan powder could reduce gene expression in the airways (bronchi and bronchioles) and alveolar walls of mice, 1.5 mg of EGFP or nontargeting siRNA dry powders was intratracheally administered to EGFP transgenic mice on days 0, 2, and 4, and the mice were sacrificed on day 6. Wild-type or EGFP transgenic mice without siRNA treatment were also



◀ **Fig. 3** (**a** and *B-1* to *-4*) Representative fluorescence images highlighting bronchioles and (**c**) comparison of fluorescence density in the epithelium of the bronchiole. (**a**) Representative image of traced epithelium in the bronchiole. (*B-1*) Bronchiole of a wild-type mouse without treatment. (*B-2*) Bronchiole of an EGFP transgenic mouse without treatment. (*B-3*) Bronchiole of an EGFP transgenic mouse treated with control siRNA. (*B-4*) Bronchiole of an EGFP transgenic mouse treated with EGFP-siRNA. (**c**) The fluorescence density of the epithelium of the bronchiole was quantified as described in the Materials and Methods. *WT siRNA (-)* wild-type mice without siRNA treatment, *EGFP-Tg siRNA (-)* EGFP transgenic mice without siRNA treatment, *EGFP-Tg nontarget siRNA* EGFP transgenic mice treated with non-targeting siRNA, *EGFP-Tg EGFP-siRNA* EGFP transgenic mice treated with EGFP-siRNA. The fluorescence density in the epithelium of the bronchiole in the EGFP-Tg EGFP-siRNA group was significantly reduced. $^{\#}P < 0.01$ *vs.* the EGFP-Tg siRNA (-) group; $^{\#\#}P < 0.05$ *vs.* the EGFP-Tg nontarget siRNA group.

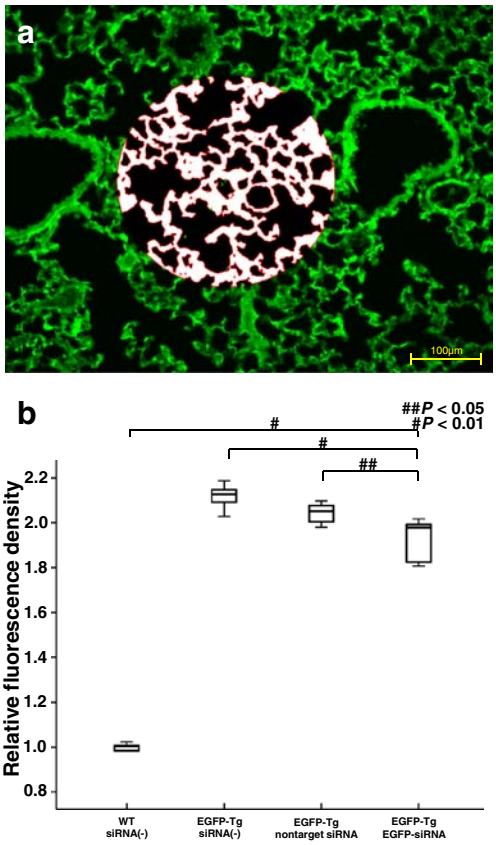


Fig. 4 (**a**) Representative image that highlights alveolar walls in *white*. Circular frames including only alveolar compartments were randomly sampled in the lung section. In each circular frame, regions whose fluorescence intensity exceeded a fixed level were highlighted to exclude alveolar air space and identify alveolar walls, and the fluorescence density was calculated in the highlighted alveolar walls. (**b**) Comparison of the fluorescence density in alveolar walls. The fluorescence density of the alveolar walls was quantified as described in the Materials and Methods. *WT siRNA (-)* wild-type mice without siRNA treatment, *EGFP-Tg siRNA (-)* EGFP transgenic mice without siRNA treatment, *EGFP-Tg nontarget siRNA* EGFP transgenic mice treated with nontargeting siRNA, *EGFP-Tg EGFP-siRNA* EGFP transgenic mice treated with EGFP-siRNA. The fluorescence density in the alveolar walls of the EGFP-Tg EGFP-siRNA group was significantly reduced. $^{\#}P < 0.01$ *vs.* the EGFP-Tg siRNA (-) group; $^{\#\#}P < 0.05$ *vs.* the EGFP-Tg nontarget siRNA group.

prepared. The regions of bronchi, bronchioles, and alveolar compartments were detected, and the fluorescence density was calculated as described in the Materials and Methods. First, we analyzed the fluorescence levels in the bronchi of the 4 groups of mice: wild-type mice without siRNA treatment, EGFP transgenic mice without siRNA treatment, EGFP transgenic mice treated with nontargeting siRNA, and EGFP transgenic mice treated with EGFP-siRNA.

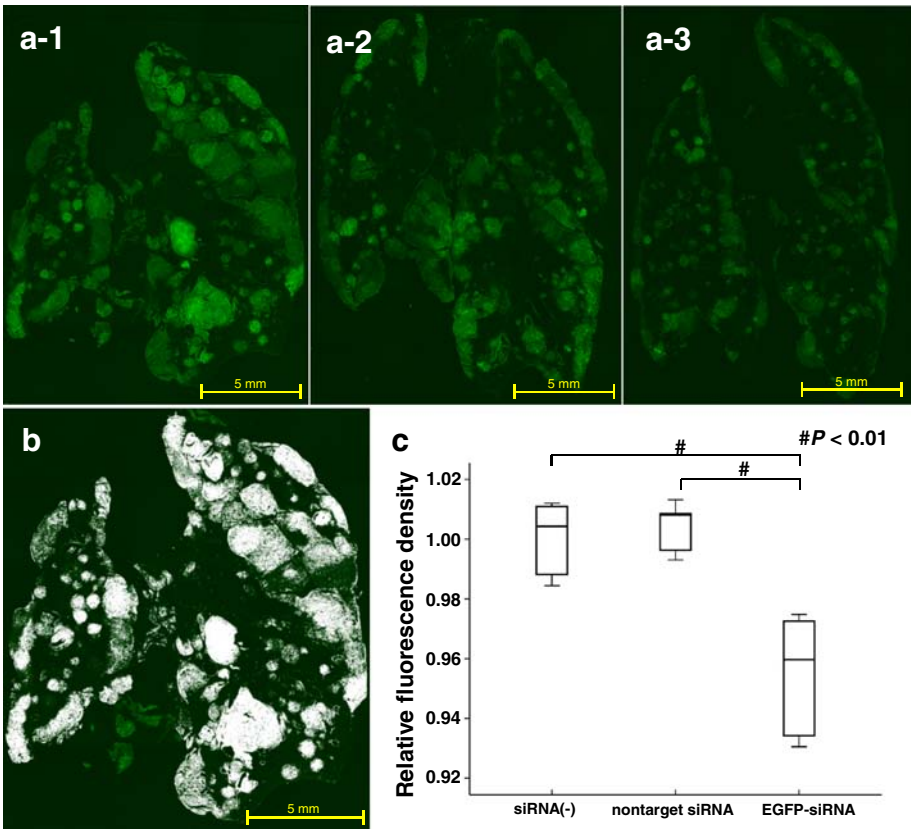


Fig. 5 (*A-1*) Representative fluorescence image of a lung section with a tumor consisting of EGFP-LLC cells from a mouse without siRNA treatment. (*A-2*) Representative fluorescence image of a lung section with tumors consisting of EGFP-LLC cells from a mouse treated with nontargeting siRNA. (*A-3*) Representative fluorescence image of a lung section with tumors consisting of EGFP-LLC cells from a mouse treated with EGFP-siRNA. (**b**) Representative image that highlights tumor regions in *white*. Regions whose fluorescence intensity exceeded a fixed level were highlighted in *white* and identified as tumors consisting of EGFP-LLC cells. (**c**) Comparison of the fluorescence densities of lung tumors consisting of EGFP-LLC cells. Fluorescence densities in such tumor regions were quantified as described in the Materials and methods. *siRNA (-)* mice without siRNA treatment; *Nontarget siRNA* mice treated with nontargeting siRNA, *EGFP-siRNA* mice treated with EGFP-siRNA. The fluorescence density of lung tumors consisting of EGFP-LLC cells in the EGFP-Tg EGFP-siRNA group was significantly reduced. $^{\#}P < 0.01$ *vs.* the EGFP-Tg siRNA (-) group and the EGFP-Tg nontarget siRNA group.

Representative fluorescence images of each group are shown in Fig. 2B-1 to B-4. The fluorescence level in the bronchus was lower in EGFP transgenic mice treated with EGFP-siRNA than in EGFP transgenic mice without siRNA treatment or treated with nontargeting siRNA. The mean fluorescence densities of bronchi in these 4 groups were then calculated (Fig. 2c). The fluorescence density of the bronchus in EGFP transgenic mice treated with EGFP-siRNA was significantly reduced compared with that of EGFP transgenic mice without siRNA treatment or treated with nontargeting siRNA and was similar to that of wild-type mice without siRNA treatment.

Next, we analyzed the fluorescence levels in bronchioles in the 4 groups of mice. Representative fluorescence images for each group are shown in Fig. 3B-1 to B-4. The mean fluorescence densities in the bronchioles were then calculated for each group (Fig. 3c). The fluorescence density of the bronchiole was significantly lower in EGFP transgenic mice treated with EGFP-siRNA than in EGFP transgenic mice without siRNA treatment or treated with nontargeting siRNA. We then analyzed the fluorescence levels in the alveolar walls of mice from each group, and the mean fluorescence densities of alveolar walls were calculated as described in the Materials and Methods (Fig. 4b). The fluorescence density of the alveolar walls was slightly but significantly lower in EGFP transgenic mice treated with EGFP-siRNA than in EGFP transgenic mice without siRNA treatment or treated with nontargeting siRNA. In fact, some high fluorescence spots were present in lung parenchyma of EGFP transgenic mice. Because similar spots were observed in a previous study (11), we believe that these spots were artifacts which were made during sectioning.

Then, we compared the histological appearance of lung sections photographed using a phase-contrast microscope between EGFP transgenic mice treated with EGFP-siRNA and those without siRNA treatment to determine whether intratracheal administration of EGFP-siRNA powder caused histological changes to the lung tissues. As shown in Fig. 6, no apparent differences in histological appearance were observed between these two groups of mice.

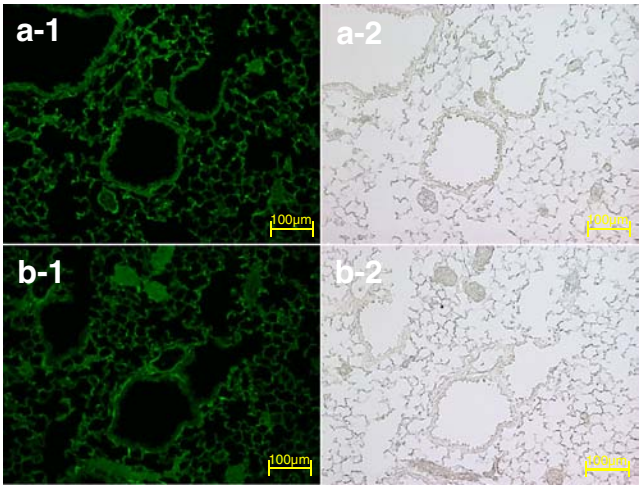


Fig. 6 Representative fluorescence and phase contrast images of identical lung sections from an EGFP transgenic mouse without siRNA treatment and that treated with EGFP-siRNA. (**a**) A fluorescence image (*A-1*) and a phase-contrast image (*A-2*) of an identical lung section from an EGFP transgenic mouse without siRNA treatment. (**b**) A fluorescence image (*B-1*) and a phase-contrast image (*B-2*) of an identical lung section from an EGFP transgenic mouse treated with EGFP-siRNA.

Suppression of EGFP Fluorescence in Metastatic Lung Tumors Consisting of EGFP-LLC Cells Following Intratracheal Administration of EGFP-siRNA/Chitosan Powder

To determine whether intratracheal administration of siRNA/chitosan powder could reduce gene expression in tumors formed in the lung, 1.5 mg of EGFP or nontargeting siRNA/chitosan dry powder were intratracheally administered on days 11 and 13 in wild-type C57BL/6 mice that were intravenously injected with EGFP-LLC cells on day 0. A separate group of mice bearing lung tumors without siRNA treatment was also analyzed. All mice were sacrificed on day 15. Representative fluorescence images of lung sections obtained from the 3 groups of mice, namely, mice without siRNA treatment, mice treated with nontargeting siRNA, and mice treated with EGFP-siRNA, are shown in Fig. 5A-1 to A-3. Visually, the fluorescence level appeared to be lower in mice treated with EGFP-siRNA than in mice without siRNA treatment or mice treated with nontargeting siRNA. To confirm this visual assessment, we calculated the fluorescence densities of the tumor regions as described in the Materials and Methods. As shown in Fig. 5c, the fluorescence densities of lung tumors consisting of EGFP-LLC cells were significantly reduced in mice treated with EGFP-siRNA compared with mice without siRNA treatment or mice treated with nontargeting siRNA.

DISCUSSION

In the present study, we prepared EGFP siRNA or nontargeting siRNA as a dry powder for inhalation in a complex with chitosan as a nonviral vector and mannitol as an excipient using the supercritical CO_2 technique. In our previous report (10), we administered 1.5 mg of a Cy5.5-labeled siRNA/chitosan/mannitol powder, which was prepared by the same supercritical fluid (SCF) process as the present study, *via* intratracheal injection with 0.25 mL air. Observation of mice with an *in-vitro* imaging system (IVIS®) revealed that the labeled powder distributed uniformly in the lungs of the mice. These siRNA/chitosan powders were able to be intratracheally and repeatedly administered into mice without increasing mortality. By performing histological quantification of EGFP fluorescence, we found that repeated intratracheal administration of EGFP-siRNA/chitosan powder reduced EGFP expression in the airways (bronchus and bronchiole) and alveolar walls of EGFP transgenic mice and in tumors consisting of EGFP-LLC cells formed in the lungs of wild-type mice. To the best of our knowledge, the present study has successfully demonstrated, for the first time, the gene silencing effect of nonviral pulmonary delivery of siRNA to each histological region of the lung and to lung tumors.

Several previous studies (11–14) have evaluated gene silencing efficacy in lung tissue sections following the nonviral delivery of siRNA to mouse lungs. However, none of these studies used the dry powder form of siRNA, and no detailed analyses of gene silencing efficacy based on different histological regions of the lung, *e.g.*, the bronchus, bronchiole, and alveolus, were performed. In addition, we found that repeated intratracheal administration of EGFP-siRNA/chitosan powder could reduce EGFP fluorescence in the bronchi of EGFP transgenic mice to a similar level in the bronchi of wild-type mice. Consistent with this, EGFP fluorescence in the bronchioles of EGFP transgenic mice treated with EGFP-siRNA was significantly lower than that of EGFP transgenic mice without siRNA treatment or treated with nontargeting siRNA; however, the level was not as low as that in the bronchioles of wild-type mice. These results suggest that the intrapulmonary delivery of this formulation of siRNA silenced gene expression more efficiently in proximal airways than in peripheral airways. Furthermore, we also found that intratracheal administration of EGFP-siRNA/chitosan powder could reduce EGFP fluorescence in the alveolar walls of EGFP transgenic mice. This reduction was minor, but statistically significant, in our histological quantification of EGFP fluorescence, suggesting that intratracheal administration of this formulation of siRNA could efficiently deliver siRNA into the alveolar spaces. Taken together, our data supported that genes expressed in the airways and alveolar walls can be targeted by intrapulmonary delivery of siRNA/chitosan powder.

Another interesting result of the present study was that intratracheal administration of siRNA/chitosan powder exhibited a gene-silencing effect in tumors formed in the lung. This effect was reported in a previous study (10); however, no studies have reported this effect using histological analysis. In

the present study, we performed histological quantification of EGFP fluorescence in tumors of EGFP-LLC cells formed in the lung and found that intrapulmonary delivery of EGFP-siRNA/chitosan powder could reduce EGFP expression in tumors. While we did not determine how the siRNA/chitosan powder was able to reach the tumors, we can speculate that there may be abundant communication pathways between tumors and the airways, allowing siRNA/chitosan powder to penetrate into tumors. Thus, our results suggested that genes expressed in lung tumors may also be targeted by intrapulmonary delivery of siRNA/chitosan powder.

We had not examined whether the powdered siRNA exerted a nonspecific gene silencing effect or a toxic effect on epithelial cells in the airway or alveolus. In the present study, we confirmed that there was no difference in EGFP intensity in the airway or alveolar epithelium between EGFP transgenic mice without siRNA treatment and those treated with nontargeting siRNA. This result suggested that the reduction of EGFP intensity observed in the airway or alveolar epithelium could be attributed to a specific gene silencing effect by siRNA targeting EGFP. In addition, we did not find any apparent morphological changes in the airway or alveolar epithelial cells of EGFP transgenic mice treated with EGFP-siRNA compared with those without siRNA treatment (Fig. 6). Although some additional experiments will be required to help clarify whether the powdered siRNA is toxic to epithelial cells in the lung, this result implied that the siRNA/chitosan powder did not induce cellular toxicity associated with morphological changes in lung epithelial cells.

For preparation of inhalable siRNA, a number of formulations, such as viral vectors (15,16), lipid vectors (17–21), and cationic polymers (22–24), have been reported. Among these, we believe that chitosan is the most attractive vector for siRNA delivery because of its biocompatibility, biodegradability, low toxicity, and ability to protect nucleic acids from enzymatic degradation (3–5,8,25–27). The biostability of siRNA has already been confirmed using the same formulation of siRNA/chitosan powder prepared in the present study (10). Thus, in this study, we successfully demonstrated that bioactive siRNA could be delivered to the airways, alveolar space, and lung tumors by intratracheal administration of siRNA/chitosan powder into mice. The results of the present study suggest that pulmonary administration of siRNA/chitosan powder may be a powerful strategy to silence genes in the lung.

CONCLUSION

Intratracheal administration of siRNA/chitosan powder resulted in significant gene-silencing effects in the bronchi, bronchioles, and alveolar walls. However, the degree of gene silencing appeared to be greater in the proximal airways than in peripheral lung tissues. In addition, we also demonstrated that intrapulmonary delivery of siRNA/chitosan powder could silence gene expression in tumors formed in the lung. These observations suggested that pulmonary administration of siRNA/chitosan powder could be a powerful strategy to target genes expressed in a wide range of respiratory diseases involving the airways, parenchyma, and lung tumors.

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