double-blind Phase IIb trial of ALN-RSV01 has been initiated in lung transplant patients to confirm and extend these findings.

3.1.2. Lung Cancer

Cancer is a major target of RNAi-based therapy, as oncogenes, mutated tumor suppressor genes, and several other genes contributing to tumor progression are potentially important targets for gene silencing by RNAi. Lung cancer is one of the most frequent tumors worldwide with regard to incidence rates and mortality. Patients with lung cancer are commonly diagnosed at an advanced stage of the disease and have limited therapeutic options. Although the knowledge regarding the genetic and molecular basis of lung cancer has regularly increased, the median survival rates of individuals with advanced lung cancer are still poor.

RNAi-based therapy is an attractive strategy for the development of more effective anticancer therapies with reduced treatment-related toxicity. The major advantage of RNAi therapeutics in cancer might be the simultaneous targeting of multiple genes belonging to different cellular pathways that are involved in tumor progression. The simultaneously inhibition of several genes would also minimize the risk of drug resistance normally encountered with small molecule-based therapies, involving siRNAs and miRNAs. There have already been significant improvements in siRNAs for primary or metastatic lung cancer treatment by targeting oncogenes such as Akt1 [9], Wilms tumor 1 (WT1) [12], overexpressed genes such as the insulin-like growth factor receptor 1 (IGF-1R) [77] , NUPR1 [53] and EZH2 [78]. Some of these studies have successfully shown the efficacy of RNAi-based therapy through intrapulmonary administration of siRNAs with non-viral vectors. Although strategies to minimize off-target and nonspecific immune stimulatory effects must be devised, these data suggest that the silencing of the target gene with siRNAs is an attractive strategy for the prevention and treatment of primary and metastatic lung cancer. There are currently some clinical trials in progress estimating the safety and efficacy of siRNA-based drugs for cancer treatment. Atu027, a siRNA-lipoplex targeted against protein kinase N3 (PKN3), prevented lung metastasis in a phase I trial of various cancer models [79]. PKN3 is a downstream effector of the phosphoinositide 3-kinase (PI3K) signaling pathway [80], which regulates diverse cellular responses, including development, growth, and survival [81]. Recently, PKN3 has also been considered as a suitable therapeutic target for modulating tumor angiogenesis because loss of function analysis with Atu027 in cultured primary endothelial cells showed an essential role of PKN3 for endothelial tube formation and migration [79]. Atu027 can be considered as a potential siRNA for preventing lung metastasis and might be suitable for preventing hematogenous metastasis combined with conventional cancer therapy.

3.1.3. Inflammatory Lung Diseases

Inflammatory lung disease, also called COPD, includes a wide range of lung ailments. These related diseases include asthma, pulmonary fibrosis, and chronic bronchitis. They are influenced by a combination of environmental, genetic, and epigenetic components [82]. COPD is a chronic inflammatory disease of the airways. This disease is hallmarked by airflow that is not fully reversible. Systemic and local airway inflammation has been implicated in the pathogenesis of COPD [83]. COPD is mainly associated with tobacco smoking, and recent studies investigating the pathophysiology of

emphysema have demonstrated that cigarette smoke can cause cells to enter cellular senescence. Smoking might cause cells to senesce due to DNA damage through increased cell turnover, which in turn leads to accelerated telomere shortening [84]. Lately, a lot of studies have investigated the role of cellular senescence in the development and progression of COPD [85]. Although several medication classes, including inhaled corticosteroids, are used for COPD treatment, none of these medications have been shown to significantly improve long-term lung function during the progression of the disease. Current interventions that have been shown to improve mortality in COPD are cessation of smoking and delivery of supplemental oxygen when hypoxemia is present.

Many people are developing COPD, and the cause of this condition is complicated and not thoroughly understood. One key factor is genetic susceptibility. Some studies have shown a large genetic contribution to the variability in pulmonary function and COPD [86,87]. Polymorphisms in multiple genes have been reported to be associated with COPD [87], such as transcription factor [e.g. nuclear factor-kappa B (NFκB)] [88], extracellular matrix (e.g., matrix metalloproteinase-12 (MMP-12)) [89,90], cytokines [e.g. tumor necrosis factor (TNF)-α] [91], chemokines [e.g. interleukins (IL)-8, IL-8 receptor and chemokine receptor (CCR)1] [92,93], and apoptosis (e.g., caspase-3 and vascular endothelial growth factor (VEGF)) [94,95]. Many of these have been identified as possible targets for therapeutic intervention using molecule inhibitors or antagonists. Although several new treatments that target the inflammatory process are now in clinical development, such as TNF-α inhibitors and I-kappaB kinase complex 2 (IKK2) inhibitors [96,97], clinical trials with siRNAs have never been performed in COPD. The delay of drug development for COPD might be due to the relatively recent emergence of research addressing the molecular basis of COPD. Furthermore, more research is needed to understand the essential molecular mechanisms about the pathogenesis of COPD and to develop monitoring techniques to support the development of RNAi therapies. Currently, no available treatments reduce the progression of COPD or suppress the inflammation in small airways and lung parenchyma. The RNAi-based approach for the key molecules also has potential implications for the treatment of COPD.

Asthma is also a chronic inflammatory disease of the airways characterized by variable and recurring symptoms and reversible airflow obstruction. The World Health Organization estimates that 300 million people are currently affected and that, by the year 2025, another 100 million will be affected by the disease [98]. Inhaled corticosteroids are very effective in mild asthma because they improve symptoms and decrease exacerbations. However, in moderate and severe asthma, inhaled corticosteroids have important therapeutic limitations. Although corticosteroids remain an important therapeutic intervention for inflammatory lung diseases, their use is not always completely effective and is associated with side effects. Due to such limitations, it is clear that there is a need for new types of medications that can treat and improve the prognosis of moderate to severe asthma.

Many target genes have been identified that participate in the pathogenesis of asthma. The most promising targets include genes coding for cytokines (IL-4, IL5, and IL-13), cytokine and chemokine receptors (IL-4 receptor and CCR3), and tyrosine kinases [spleen tyrosine kinase (Syk) and LCK/YES-related novel tyrosine kinase (Lyn)], as well as for transcription factors [signal transducers and activators of transcription 1 (STAT1), STAT6, GATA3, and NFκB] that are involved in asthma [19,99,100]. The genes that have been assessed as siRNA targets for the treatment of asthma in preclinical models are reported [101]. Currently, in a clinical trial for asthma, Excellair™ (ZaBeCor, Bala Cynwyd, PA,

USA), a siRNA that targets Syk, is being used. The kinase is involved in signaling from a B cell receptor and is a key regulator of downstream signaling cascades that ultimately lead to the activation of several pro-inflammatory transcription factors. It has been reported that antisense oligonucleotides administered by aerosol were potent to decrease Syk expression, mediator release from alveolar macrophages, and Syk-dependent pulmonary inflammation [102]. Moreover, inhibition of inflammatory mediators was shown in a study using siRNA targeting Syk in airway epithelial cells [103]. Following the successful results of the company's Phase I clinical trial, a Phase II trial for its asthma drug candidate Excellair™ has already been initiated. Some of the current treatments for asthma and other inflammatory conditions, such as TNF-α inhibitors or leukotriene inhibitors, inhibit only one of the mediators of inflammation. In contrast, siRNA targeting Syk seeks to inhibit an initial signaling step of inflammation and, thereby, prevent the release of multiple inflammatory mediators. Overall, recent progress of siRNAs to the lungs has also improved the therapeutic feasibility of RNAi for inflammatory lung diseases. The rapid progress will put siRNA-based therapeutics on a fast track to the clinic.

3.2. Therapeutic microRNA/Anti-microRNA for Lung Diseases

MiRNAs are small endogenous noncoding RNAs that regulate gene expression by repressing translation or promoting the degradation of their target mRNA. MiRNAs regulate gene expression by binding to the 3′ untranslated region (UTR) of their target mRNAs and mediating mRNA degradation or translational inhibition. In the human genome, transcripts of approximately 60% of all mRNAs are estimated to be targeted by miRNAs [104]. According to their function, miRNAs play an important role in cellular processes as development, proliferation, and apoptosis of pulmonary pathologies [105]. A growing number of miRNAs have been shown to be involved in different lung diseases. This evidence makes miRNAs a promising technology for current and future therapeutic development. We discuss the role of some miRNAs in various lung diseases as well as the possible future of these discoveries in clinical applications. Table 2 shows the summary of miRNAs in therapeutic development. At this point, a miRNA-based therapy has already entered a phase II clinical trial.

Table 2. miRNAs in therapeutic development.

miRNA	Disease	Stage of clinical trial	Reference
	miRNA replacement		
let-7	Lung cancer	Preclinical	[106]
miR-34	Lung cancer, Prostate cancer	Preclinical	[107,108]
miR-29	Cardiac fibrosis	Preclinical	[109]
	miRNA antagonists		
miR-122	Hepatitis C virus	II	[110]
miR-208/499	Chronic heart failure	Preclinical	[111]
miR-15/195	Post-myocardial infarction remodeling	Preclinical	[112,113]
miR-206	Amyotrophic lateral sclerosis	Preclinical	[114]
miR-451	Myeloproliferative diseases	Preclinical	[115]

3.2.1. Role of microRNA in Inflammatory Lung Diseases

There is evidence that upregulation or downregulation of miRNAs is critical for lung homeostasis and, thus, may contribute to the development of pathological pulmonary conditions. Many studies have focused on the role of miRNAs in inflammatory lung diseases, such as COPD [116,117], pulmonary fibrosis [118–121], and asthma [122–125] (Table 3).

Table 3. miRNAs in inflammatory lung diseases.

Lung diseases	Expression of specific miRNA		Reference
COPD	miR-223/1274a	↑	[126]
	let-7	↓	[117]
	miR-1	↓	[116]
	miR-146a	↓	[127]
	miR-150	↓	[117]
Asthma	miR-21	↑	[123]
	miR-126	↑	[124]
	miR-155	↑	[122]
	miR-133a	↓	[125]
Pulmonary fibrosis	miR-21	↑	[118]
	miR-155	↑	[128]
	let-7d	↓	[119]
	miR-29	↓	[120]
	miR-200	↓	[121]
Smoking-related miRNA	*let-7*	↓	[129]
	miR-10a	↓	[129]
	miR-34	↓	[129]
	miR-123	↓	[129]
	miR-145	↓	[129]
	miR-150	↓	[117]
	miR-199b	↓	[130]
	miR-218	↓	[130]
	miR-222	↓	[117,129]

The pathogenesis of COPD is attributed to not only chronic inflammation in the airways but also systemic inflammation [131]. Cigarette smoking is the main risk factor for the development of COPD. Smoking has been shown to cause biological change in the gene expression of the lungs [132], and there are some reports about smoking-related miRNAs [117,129,130]. However, there are few reports that focus on the miRNAs related to the pathogenesis of this disease with systemic inflammatory components. Recent study on pulmonary fibroblasts of COPD patients presents less expression of miR-146a after stimulation with proinflammatory cytokines when compared with non-COPD subjects with similar smoking histories [127]. The downregulation of miR-146a resulted in a prolonged mRNA half-life of cyclooxygenase-2, thus increasing prostaglandin E2 in fibroblasts from COPD subjects.

Moreover, Ezzie *et al.* researched the difference of miRNA profiles expressed in the lungs of smokers with and without COPD. They concluded that miR-223 and miR-1274a were the most affected miRNAs in subjects with COPD [126]. Yet, COPD is a complex, multi-component, and heterogeneous disorder with a number of different pathological processes and subgroups with their own characteristics and natural history [133]. A better understanding of the complexity of the disease and potential clinical relevance of the identified miRNAs is needed.

Pulmonary fibrosis can be caused by an identifiable irritation to the lungs, but, in many cases, the cause is unknown, and the therapeutic possibilities are limited. Cigarette smoking is one of the most recognized risk factors for the development of pulmonary fibrosis. This disorder is mainly accompanied by increased expression of the key fibrotic mediator transforming growth factor β (TGF-β) and other cytokines produced at the lesion of active fibrosis [128]. Recently, it was reported that miRNAs may play an important regulatory role in the pulmonary fibrotic change in the lungs. The downregulation of *let-7d* in idiopathic pulmonary fibrosis (IPF) resulted in increased collagen deposition and alveolar septal thickening [119]. In addition, Liu *et al.* reported that the oncogenic miR-21 was found to be upregulated in IPF patients and in the murine lungs with bleomycin-induced fibrosis [118]. Although these miRNAs may be potential therapeutic targets because their expression is related to the regulation of TGF-β, the factor is necessary but not sufficient for pathologic fibrosis of the lungs. Pulmonary fibrosis is also a complicated illness that can have many different causes.

Focus on the role of miRNAs in asthma has recently increased. Asthma is an inflammatory disease of the airway that is characterized by an abnormal response of T helper-2 (Th2)-type CD4+T lymphocytes against inhaled allergens [134]. In a different asthmatic mouse model, there was an observed increase in the expression of miR-21 in the lungs [123]. This report might contribute to the understanding of the inflammatory mechanism in the airway through the inhibition of IL-12, favoring the Th2 lymphocyte response. A toll-like receptor 4 (TLR4)-induced Th2 lymphocyte induces high expression of miR-126, and selective blockade of miR-126 suppressed the asthmatic phenotype [124]. In addition, airway remodeling is a characteristic feature of asthma and has important functional implications. Rodriguez *et al.* have shown that miR-155 is related to the development of inflammatory infiltration into the lung and airway remodeling [122]. Thus, some studies present a functional connection between miRNA expression and asthma pathogenesis and suggest that targeting miRNAs in the airways may lead to anti-inflammatory treatments for allergic asthma. Despite the evidence from experimental models, the expression profiling of miRNAs in airway biopsies from patients with mild asthma before and after treatment with inhaled corticosteroids and in healthy volunteers revealed no differences in miRNA expression [135]. Further investigations about the role of miRNAs related to asthma pathogenesis are required.

Although the basic evidence of miRNA biology is still providing new insights, applications of miRNA-based therapy for inflammatory lung diseases are less advanced than those for lung cancer [136]. One reason for this could be that the disease heterogeneity is caused by the effects of many environmental air pollutants, including smoke and volatile organic compounds. The presence of several risk factors makes the understanding of the pathogenesis of inflammatory lung diseases complicated. Understanding the role that miRNAs play in the modulation of gene expression, leading to sustain the pathogenesis of lung diseases, is important for the development of new therapies that focus on the prevention of disease progression and symptom relief.

3.2.2. microRNA/Anti-microRNA Delivery for Lung Cancer Therapy

Given the significant roles that miRNAs play in multiple pathways of lung carcinogenesis, increasing efforts are dedicated to the research and development of miRNA-based therapies, including restoring functions of tumor suppressive miRNAs or inhibiting oncogenic miRNAs. The development of miRNA-based therapies for lung cancer is growing prosperously with the help of new RNAi technologies. Compared to siRNA-based therapies, which are already in clinical trials, miRNAs are less toxic and have the potential to target multiple genes. The difficulty associated with miRNA delivery is mainly equal to that of siRNAs. The critical problems for the development of this therapy are effective delivery into target sites, potency of the therapy, and elimination of off-target effects [137].

There are two strategies as the therapeutic applications of miRNAs for lung cancer [138]. One strategy is miRNA replacement therapy, which involves the re-introduction of a tumor suppressor miRNA mimic to restore a loss of the function. MiRNA mimics are synthetic RNA duplexes designed to mimic the endogenous functions of miRNAs with chemical modifications for stability and cellular uptake. The concept of miRNA replacement therapy is most exemplified by the *let-7* miRNA. *let-7* is a tumor-suppressor miRNA in non-small-cell lung cancer that inversely correlates with the expression of the RAS oncoprotein, a key cancer gene [139]. Intranasal administration of *let-7* mimic into mouse models of lung cancer significantly reduced tumor growth, suggesting that miRNA replacement therapy is indeed promising [106,140,141]. Another miRNA that shows the value of miRNA replacement is provided by miR-34a [107,142]. Local and/or systemic delivery of a synthetic miR-34a mimic led to accumulation of miR-34a in the tumor tissue and inhibition of lung tumor growth. Lately, Ling *et al.* also showed that tumor suppressor miR-22 exhibited anti-lung cancer activity through post-transcriptional regulation of ErbB3 [143]. Thus, therapeutic miRNA mimics have a powerful potential by attacking multiple genes relevant to several diseases. However, it is necessary to pay attention to the potential toxicity in normal tissues under conditions in which the therapeutic delivery of miRNA mimics will lead to an accumulation of exogenous miRNAs in normal cells [138]. Although the assumptions are well founded, there is still insufficient evidence for toxicity caused by miRNA mimics. Indeed, several *in vivo* studies failed to reveal side effects caused by the miRNA mimics and suggested that delivery of miRNA mimics to normal tissues was well tolerated [107,141]. It will be important to research miRNA mimic-induced effects in normal cells and to carefully assess toxicity before using them in clinical practice.

The second strategy is directed toward a gain of function and aims to inhibit oncomiRs by using anti-miRNAs. Chemical modifications, such as 2'-*O*-methyl-group and locked nucleic acid (LNA), would increase oligo stability against nucleases [144]. Antisense oligonucleotides contained in these modifications are termed antagomirs or "LNA-antimiRs" [144,145]. They are oligonucleotides with sequences complementary to the endogenous miRNA and inhibit the specific miRNA function. An LNA-antimiR against miR-122 has been shown to effectively silence miR-122 in non-human primates [145], and the findings support the potential of these compounds as a new class of therapeutics. Moreover, it has also been reported that anti-miR-150 delivered into lung tumor xenografts in mice led to inhibited tumor growth [146]. Relative to studies on miRNA mimics, studies with antisense oligonucleotides have shown effective evidence with naked oligonucleotides. This illustrates the potential of chemical modifications of oligonucleotides to improve their stability, resistance to RNase, and pharmacologic

properties. Therefore, inhibition of miRNA function by chemically modified antimiR oligonucleotides has become an important and widely used approach. Recent data from the first phase II study in patients with chronic HCV infection treated with the LNA-modified antimiR-122 showed that this compound was well tolerated and provided continuing viral suppression.

An increasing number of studies have examined the therapeutic potential of miRNAs. Recently, the evidence of roles for miRNAs in determining drug resistance has emerged [147]. Cytotoxic and molecular target drugs have been widely used in the treatment of advanced lung cancer; unfortunately, many cases are still refractory to chemotherapy. In this situation, combining miRNA mimics or antimiR with chemotherapy may potentiate the efficacy of the cancer treatment in the future. In addition, miRNAs related with cancer stem cells may significantly broaden the field of miRNA-based therapy and suggest that miRNAs can be potential tools to kill cancer cells associated with therapy resistance, recurrence, and metastasis [108,148]. Hence, the main challenge is the successful delivery and chemical modifications of the therapeutic miRNAs to the target tissue without harming normal tissues.

4. Conclusions and Prospects

RNAi-based approaches provide a promising therapeutic modality for the treatment of various lung diseases. One of the greatest challenges in RNAi-based therapy continues to be the delivery method of the therapeutic siRNAs and miRNAs to the target cells. Pulmonary delivery applications are very attractive, since they tend to be non-invasive, are locally restricted, and can be administered by the patient. A realistic therapeutic intervention, such as aerosolization, can enhance drug delivery to the site of action and decrease systemic exposure of the patient to the therapy, thereby reducing off-target effects. The advancement of pulmonary siRNA delivery to the clinic illustrates that RNAi-based therapy holds a central place in the future treatment of lung diseases. On the other hand, miRNAs have the opportunity to target multiple genes in a fine-tuned manner, and the miRNA-based therapy will provide an attractive anti-tumor and anti-inflammatory approach for various lung diseases. In particular, anti-miRNA therapy by chemically modified antimiR oligonucleotides has become a potential therapy for lung diseases because the oligonucleotides can be successfully delivered without delivery vectors. Increased evidence has indicated that miRNAs fulfill causative roles in a variety of lung diseases and have prompted investigations into their potential as therapeutic targets. Further understanding of the detailed mechanisms of RNAi-based therapy and investigations of more effective delivery methods are required for future development. These novel approaches could open new avenues for various lung diseases and improve the clinical outcome of the patients.

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Conflict of Interest

The authors declare that there are not conflicts of interest to report.

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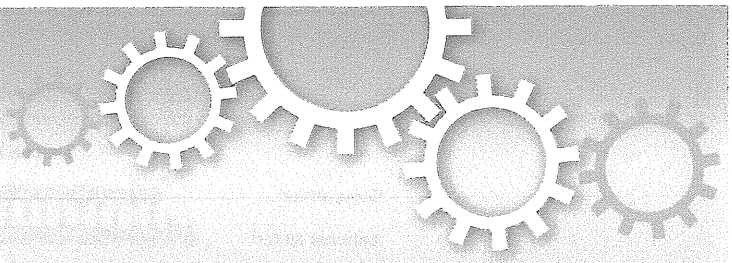

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A novel platform to enable inhaled naked RNAi medicine for lung cancer

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Small interfering RNA (siRNA)-based therapeutics have been used in humans and offer distinct advantages over traditional therapies. However, previous investigations have shown that there are several technical obstacles that need to be overcome before routine clinical applications are used. Currently, we are launching a novel class of RNAi therapeutic agents (PnkRNA™, nkRNA®) that show high resistance to degradation and are less immunogenic, less cytotoxic, and capable of efficient intracellular delivery. Here, we develop a novel platform to promote naked RNAi approaches administered through inhalation without sophisticated delivery technology in mice. Furthermore, a naked and unmodified novel RNAi agent, such as ribophorin II (RPN2-PnkRNA), which has been selected as a therapeutic target for lung cancer, resulted in efficient inhibition of tumor growth without any significant toxicity. Thus, this new technology using aerosol delivery could represent a safe, potentially RNAi-based strategy for clinical applications in lung cancer treatment without delivery vehicles.

RNA interference (RNAi) is a post-transcriptional gene-silencing mechanism. Small interfering RNA (siRNA) must be dissociated into their component single strands to act as a guide for the RNA-induced silencing complexes, which are the protein complexes that repress gene expression[1]. The development of siRNA technology has opened an avenue of opportunity to study gene function, as well as the possibility of novel forms of therapeutic intervention in several genetic diseases. In fact, siRNA-based therapy has enormous potential for the treatment of several diseases through either local or systemic administration of siRNAs that are being tested in experimental animal models or in clinical development[2]. Oncology is one of the medical fields that can benefit most from this powerful therapeutic strategy because this approach can modulate the expression of target genes involved in tumor initiation, growth, and metastasis[3].

However, the clinical application of siRNAs has been impaired by problems related to their delivery, low biological stability, off-target gene silencing, and immunostimulatory effects[4,5]. Indeed, naked siRNAs are promptly degraded by nucleases in serum and extracellular fluids, and chemical modifications at specific positions or formulations with delivery vehicles have been shown to improve stability. However, these may attenuate the suppressive activity of siRNAs[6]. Furthermore, the cost of large-scale production is another obstacle to the clinical application of siRNAs[7]. For this reason, their translation to the clinical setting is dependent upon the development of an efficient delivery system that is able to improve the pharmacokinetic and biodistribution properties of siRNAs.

Recently, engineered designs, such as aptamer-siRNA chimeras and transferring-decorated nanoparticles, have continued to dramatically improve the precision of delivery for RNAi agents[8]. Advances in RNAi-based therapeutics may require new biochemical technologies to maximise drug potency while minimising off-target toxicity and immunogenicity. Meanwhile, we have already reported a novel class of RNAi therapeutic agents (PnkRNA, nkRNA) and evaluated their effectiveness[9]. We showed that PnkRNA and nkRNA directed against transforming growth factor (TGF)-β1 ameliorate outcomes in mouse models of acute lung injury and pulmonary fibrosis. This novel class of RNAi agents was synthesised on solid phase as single-stranded RNAs (ssRNAs) that self-anneal into a unique helical structure containing a central stem and two loops following synthesis (Fig. 1). The production of the novel RNAi agents is simple; because PnkRNA and nkRNA are synthesised as ssRNAs that spontaneously self-anneal, low-cost, large-scale production is possible. These novel RNAi agents have showed significant effectiveness in disease models and also superior resistance against nuclease degradation compared to

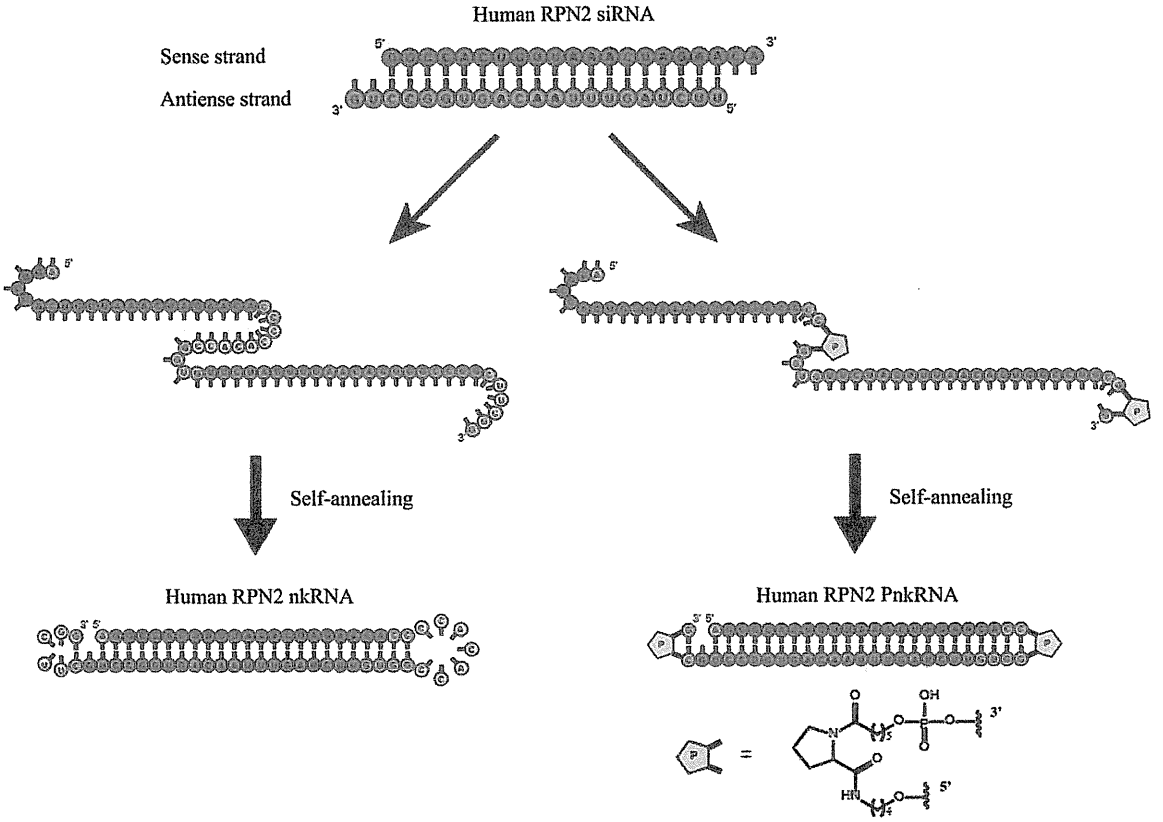

Figure 1 | Structure of novel RNAi agents. Both nkRNA and PnkRNA were prepared as single-stranded RNA oligomers that then self-anneal, as shown. Nucleotides in red indicate the sense strand of the target (RPN2); nucleotides in blue indicate the antisense strand; and nucleotides in green and yellow indicate the loop cassettes. P indicates a proline derivative.

canonical siRNAs. Additionally, by evaluating the induction of proinflammatory cytokines, our previous results suggest that none of the platforms were immunotoxic[9]. Thus, the novel RNAi therapeutic agents are safe and might be employed in clinical applications because they address several issues in siRNA-based therapy.

Lung cancer is the leading cause of cancer-related death in the world. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers. Approximately 70% of all newly diagnosed patients present with local advanced or metastatic disease and require systemic chemotherapy[10,11]. Although NSCLC patients with epidermal growth factor receptor (EGFR) mutations initially respond to EGFR tyrosine kinase inhibitors[12], most patients experience a relapse within 1 year. Despite the development of novel molecular therapies[13], the prognosis of lung cancer is still poor and shows a median survival time of approximately 18 months in the operable stages. Hence, novel and more effective approaches are needed for the treatment of advanced lung cancer. Lung diseases in general are attractive targets for siRNA-based therapeutics because of their lethality and prevalence. In addition, the lung is anatomically accessible to therapeutic agents via the intrapulmonary route. Accessibility is a key requirement for successful RNAi-based *in vivo* and clinical studies, and this characteristic offers several important benefits over systemic delivery, including the use of lower doses of siRNAs, the reduction of undesirable systemic side effects, and improved siRNA stability due to the lower nuclease activity in the airways compared to the serum[14]. The direct administration of siRNAs into the target organs is a promising strategy for overcoming the problems of intravenous administration[15]. On the basis of these promising findings, we explored the effectiveness of this novel class of RNAi therapeutic agents in lung cancer. In the current study, we prepared inhaled novel RNAi agents directed against luciferase and human-ribophorin II (RPN2) as candidate genes, and we compared their inhibitory activity to canonical siRNAs using *in vitro* assays and animal models of lung cancer. Furthermore, using this new technology, lung cancer xenograft studies showed that the aerosol delivery of a naked novel RNAi agent significantly inhibited tumor growth.

Results

***In vitro* stability of novel RNAi agents.** The stability of the novel RNAi agents, PnkRNA and nkRNA, against degradation by ribonucleases was compared with that of siRNA. Each RNAi agent was incubated in the presence of ribonucleases, and the degree of degradation was followed overtime. siRNA was degraded after 5 min of incubation with ribonucleases, but both PnkRNA and nkRNA remained intact after 15 min of incubation (Supplementary Fig. 1). The novel RNAi platforms were more resistant to degradation than siRNA.

Distribution of fluorescent siRNA after inhalation. The inhaled administration of fluorescently labeled siRNA by MicroSprayer™ enabled the efficient intracellular distribution of lung cancer cells throughout the lung parenchyma. The efficient and homogeneous distribution of the RNAi agent to most parts of the lung tissue is a prerequisite for studying target-specific RNAi efficacy. The endotracheal application of suspended RNAi agents by a MicroSprayer™ can result in nanoparticle deposition in smaller airway and peripleural tumor cells (Fig. 2a,b).

Monitoring luciferase inhibition of the inhaled novel RNAi agents' delivery system *in vivo*. Mice with an intravenous injection of

A549-luc-C8 cells on day 0 were imaged twice a week up to day 28. In all mice, measurable lung tumors could be calipered within 2 weeks using this cell line. Four weeks after tumor injection, the observed patterns indicated lesions developing in the lungs of the mice. To estimate whether the aerosol delivery of novel RNAi agents had a valid gene-silencing effect on the lung tumors, the mice were treated with a luciferase siRNA, PnkRNA, and nkRNA, each of which was compared with the control siRNA. The activities of the siRNA and novel RNAi platforms are shown. On the next day, in mice receiving luciferase siRNA and novel RNAi agents, bioluminescence was inhibited by 50–60% in the whole body when compared with bioluminescence before treatment. On the other hand, the bioluminescent signals in the mice that were treated with the control siRNA had increased (Fig. 2c,d). In addition, we found that the RNAi effect of the novel platform would continue for at least five days (supplementary Fig. 2).

***In vitro* suppressive effect of novel RNAi agents for RPN2.** To screen for target genes showing the growth inhibition of A549-luc-C8 cells, RPN2 was selected as a target gene. A549-luc-C8 cells expressed RPN2 mRNA at high levels, as evaluated by real-time RT-PCR, and RPN2 protein expression on immunohistochemical staining was detected in the cytoplasm of cancer cells in the A549-luc-C8 xenograft model (Fig. 3a). To monitor cell growth and

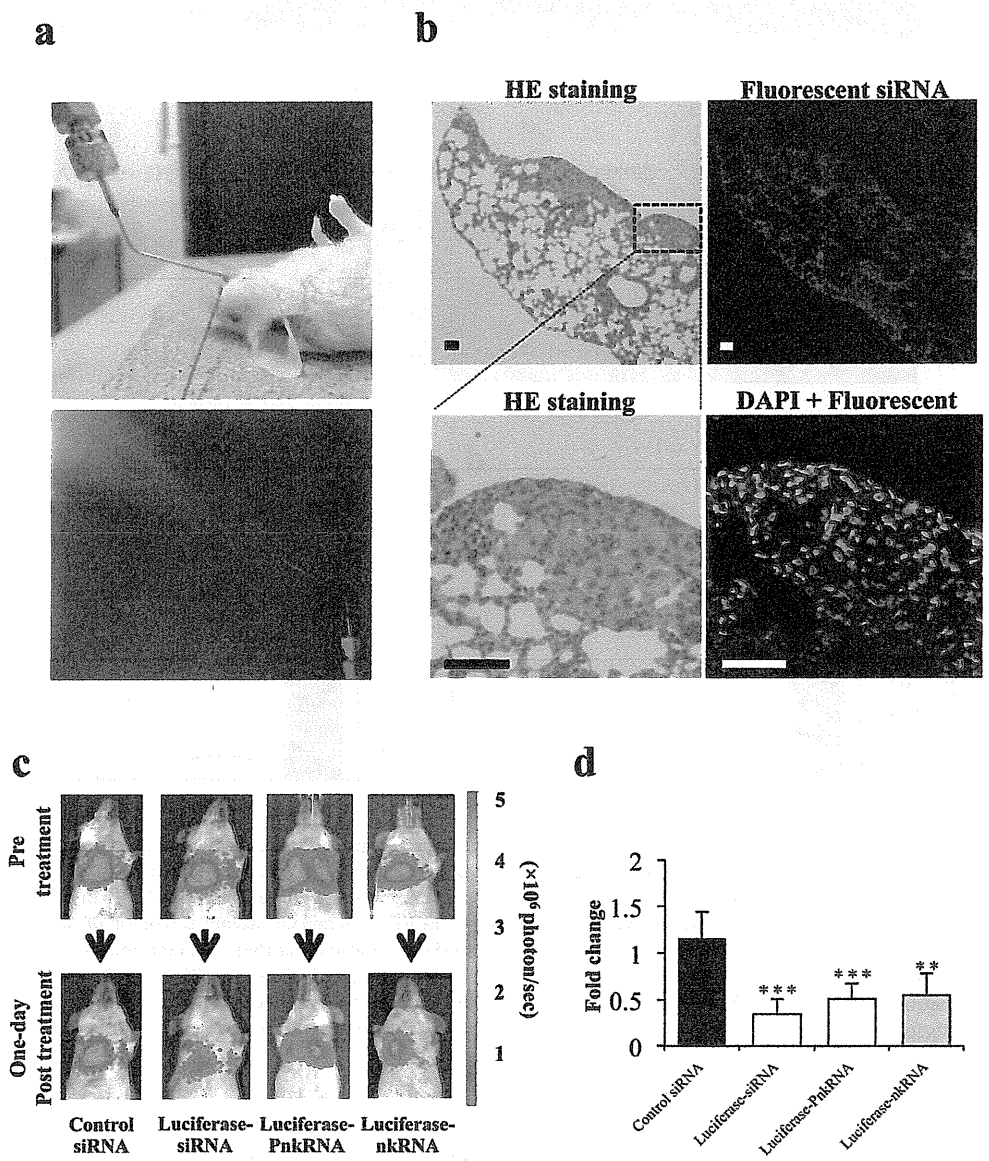

Figure 2 | Inhaled single administration study with novel RNAi platforms against luciferase gene *in vivo*. (a) Intratracheal delivery route: RNAi therapeutic agents are sprayed directly from the mouth into the lungs using a MicroSprayer™ aeroliser. (b) Distribution of fluorescent siRNA in the lungs after inhalation. A sufficient pulmonary distribution of aerosolised siRNA was attained in mice by MicroSprayer™. In addition, intracellular fluorescent staining in lung cancer cells and bronchial epithelial cells was occasionally observed (magnified image, DAPI + Fluorescent siRNA). The scale bars indicate 50 μm. HE, Haematoxylin-eosin. (c) Monitoring luciferase inhibition *in vivo* with bioluminescent imaging. Representative images pre-treatment and on the first day post-treatment. (d) Normalised fold change (one day post-treatment/pre-treatment) of bioluminescence emitted from the whole bodies of mice. The data represent the means ± SD (n = 4). Statistical analysis was performed by the Bonferroni multiple-comparison test. ***, $P < 0.001$ versus control siRNA group. **, $P < 0.01$ versus control siRNA group.