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REVIEW ARTICLE

RNAi therapeutic strategies for acute respiratory distress syndrome

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Acute respiratory distress syndrome (ARDS), replacing the clinical term acute lung injury, involves serious pathophysiological lung changes that arise from a variety of pulmonary and nonpulmonary injuries and currently has no pharmacological therapeutics. RNA interference (RNAi) has the potential to generate therapeutic effects that would increase patient survival rates from this condition. It is the purpose of this review to discuss potential targets in treating ARDS with RNAi strategies, as well as to outline the challenges of oligonucleotide delivery to the lung and tactics to circumvent these delivery barriers. (Translational Research 2019; 214:30–49)

INTRODUCTION

Acute respiratory distress syndrome (ARDS) develops from direct or indirect injury to the lung, from conditions including sepsis, severe trauma, bacterial/viral pneumonia, and aspiration.[1,2] The disease occurs in approximately 80 out of 100,000 individuals per year in the United States, with a mortality rate of up to 40% when severe ARDS is developed.[3,4] Interestingly, studies have shown that ARDS survivors usually only demonstrate mild deficiencies in pulmonary functions and physical capacity following the first 3 months postrecovery.[5,6] Amazingly, patients that survive typically regain normal pulmonary function and capacity as early as 6-12 months postrecovery; unfortunately, long-term pulmonary structural changes are difficult to evaluate.[7–9] This review will focus on the pathophysiological characteristics of ARDS, current approaches for treatment, methods for utilizing RNA interference (RNAi) therapeutics, and challenges for treating ARDS using these methods.

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Submitted for Publication May 2, 2019; received submitted July 22, 2019; accepted for publication July 23, 2019.

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1931-5244/$ - see front matter

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https://doi.org/10.1016/j.trsl.2019.07.011

Molecular etiology of ARDS. Clinical diagnosis of ARDS is characterized by an abrupt onset of severe hypoxia and accumulation of pulmonary edema.[10] ARDS can result from direct injury to the lung ($ARDS_{direct}$) or extrapulmonary injury, nonpulmonary sepsis, and indirect trauma to the lung ($ARDS_{indirect}$).[11] Cellular characteristics of ARDS include loss of the alveolar-capillary membrane integrity, excessive transendothelial, and transalveolar neutrophil migration, and release of proinflammatory cytotoxic mediators, resulting in further disruption of the epithelial/endothelial barrier[12–14] (Fig 1).

The alveolar-capillary unit is primarily composed of alveolar epithelial and pulmonary endothelial cells and which are both prone to injury in both $ARDS_{direct}$ and $ARDS_{indirect}$. Endothelial dysfunction is characterized by acquisition of proadhesive and proinflammatory phenotypes, and loss of endothelial barrier integrity, leading to inflammatory cell infiltration and plasma extravasation into the interstitium.[15] Similar aberrant responses from activated epithelial cells, coupled with their diminished ability of fluid clearance, paves the way for a massive influx of inflammatory cells and protein-rich fluid from the interstitium into the alveolar space with severe consequences for pulmonary gas exchange.[16,17] These processes are further augmented by apoptosis and necrosis of these (endothelial and

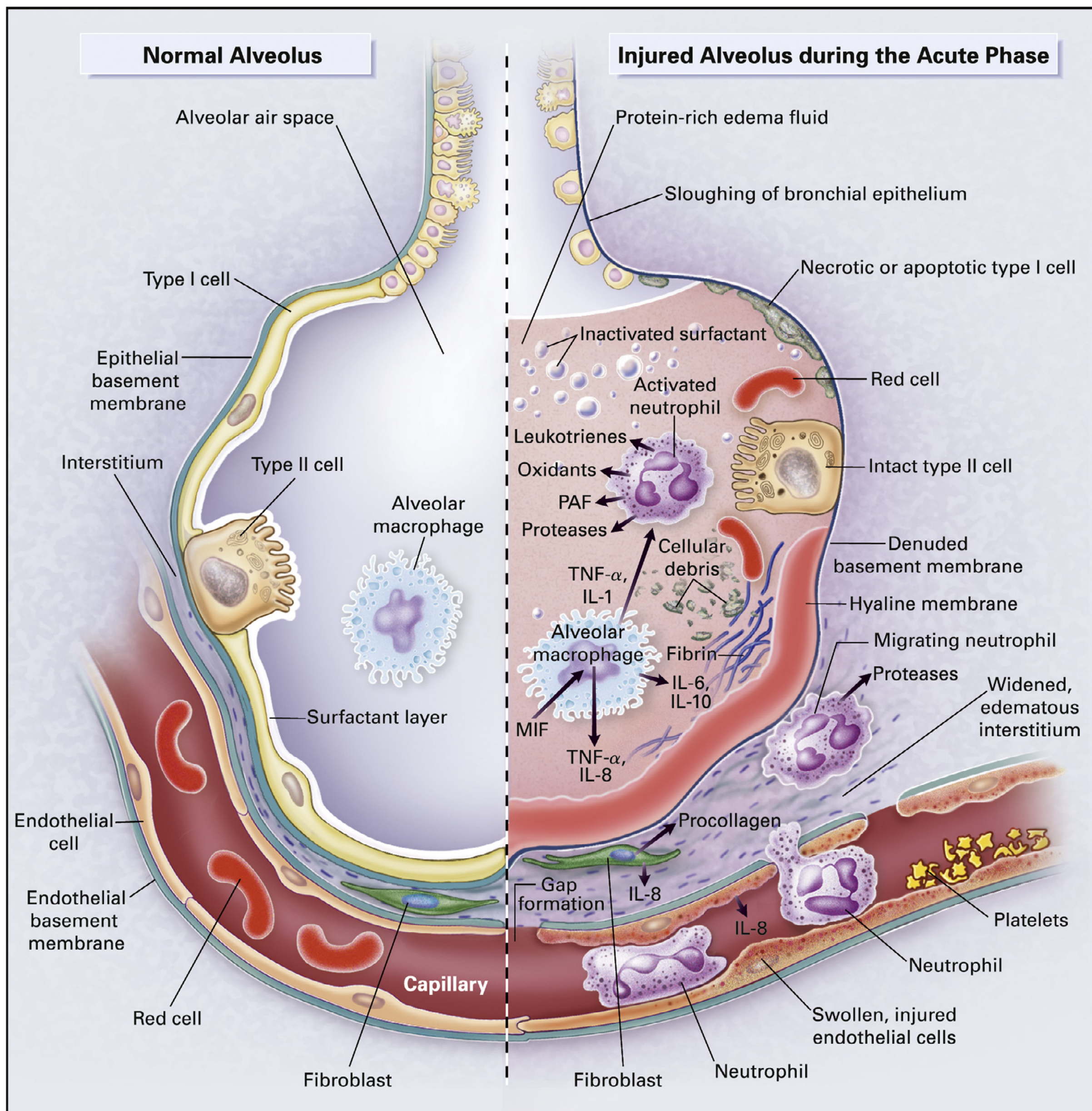

Fig 1. Effects of ARDS on lung alveoli. This figure shows the molecular physiology of a healthy alveolus (left) compared to a patient with ARDS (right). The healthy alveolus is lined with pulmonary surfactant and free of pulmonary edema; however, ARDS patients experience impaired gas exchange due to pulmonary edema, resulting from massive influx of fluid and inflammatory cells secondary to loss of endothelial and epithelial barriers. The shedding of the surfactant layer triggers immune responses by macrophages and neutrophils, which further augments these processes. The figure illustrates some of the important regulatory signaling pathways activated by ARDS, which are discussed as potential therapeutic targets. From Ware and Matthay.[14] (Color version of figure is available online.)

epithelial) cells, which promote further inflammation and activation of coagulation cascades on denuded surfaces in a continuous cycle that amplifies and exacerbates ARDS.[15,16,18,19]

While the disease can be caused by a multitude of different factors, the molecular mechanisms have invited much investigation leading to two prevailing hypotheses to explain the etiology of ARDS: the neutrophil hypothesis and the epithelial/endothelial hypothesis.[20] The neutrophil hypothesis states that neutrophils are activated in the bloodstream and in the lung by proinflammatory mediators and chemokines. Prolonged neutrophil activation causes rapid release of reactive oxygen species (ROS),[21−23] preventing transition from a proinflammatory

to an anti-inflammatory environment.[24,25] The epithelial/endothelial hypothesis states that the apoptosis of lung epithelial/endothelial cells results in loss of epithelial-endothelial barrier function.[20,26] Pathophysiological characteristics indicate that both hypotheses are implicated in ARDS development. Neutrophil recruitment and epithelial/endothelial cell death both play a key role in $ARDS_{direct}$, while epithelial/endothelial cell death is more prevalent in $ARDS_{indirect}$.[20,25,27,28] While a higher degree of epithelial damage has been noted in lungs from septic patients compared to endothelial cells, it is attributed not to the lesser degree of damage, but to higher regenerative capacity of endothelial cells.[29] Consistent with this, endothelial cells from endotoxemic mice have been shown to possess regenerative capacity, which relies on activation of Fox1M transcription factor.[30]

Endothelial barrier integrity is important because it restricts the movement of proteins and fluids into the interstitium. Endothelial cells form a continuous monolayer that creates an endothelial-capillary barrier via tight and adheren junctions.[31,32] Endothelial cells can cause the dysfunction of their junctions by secreting tumor necrosis factor α (TNF-α) to increase the influx of pulmonary edema, which acts by phosphorylating adheren junction proteins.[33,34] Interendothelial gaps can also result from cytoskeleton contraction due to phosphorylation of myosin light chain (MLC).[35] Influx of Ca^{2+} has been shown to increase vascular permeability and allow for migration of neutrophils across the alveolocapillary barrier.[36] The production of free radicals by neutrophils and other polymorphonuclear leukocytes contributes to vascular leakage.[37,38]

Epithelial cell death is another mechanism in ARDS that greatly contributes to disease progression and severity.[39] Lung epithelial cells are the cell type that mainly undergo apoptosis in ARDS, a process which has been detected as quickly as 6 hours in postinjury mice.[39,40] In healthy lung tissue, apoptosis is prevented by surfactant protein A released by type II pneumocytes.[41,42] Unfortunately, the bronchoalveolar (BAL) fluid of ARDS afflicted lung contains a reduced concentration of surfactant protein A, which greatly increases epithelial apoptosis propensity.[43] Epithelial cell death can be triggered extrinsically or intrinsically. The intrinsic pathway is initiated by Bcl-2 proteins which increase the permeability of the outer mitochondrial membrane, resulting in the irreversible release of cytochrome c into the cytosol.[44] The extrinsic pathway is initiated by ligation of cell-surface death receptors and their ligands.[44] One of the most important death receptors is Fas, which interacts with soluble FasL or FasL expressed on lymphocytes to induce apoptosis. Onset of ARDS results in increased expression of Fas on alveolar epithelial cells and an increase in FasL concentration in BAL fluid.[20,45–47] Fas silencing in lung epithelium potentially moderates ARDS by decreasing lung apoptosis and reducing the severity of pulmonary histological changes.[20] The intrinsic and extrinsic pathways result in activation of caspases that cause irreversible DNA fragmentation and phosphatidylserine externalization.[44] Epithelial apoptosis results in disruption of the alveolar barrier, allowing pulmonary edema to flood the alveolar spaces. This hypothesis has been supported by the detection of increased Fas expression in lung epithelium.[26,39,48] Neutrophil accumulation in the lung during ARDS also increases epithelial cell death and inflammation.[39,48] Neutrophils release proinflammatory factors and ROS, triggering lung epithelial cell apoptosis. These molecular mechanisms provide many potential therapeutic targets that can be targeted with RNAi.

ARDS diagnosis is solely dependent on clinical criteria due to the impracticality of obtaining direct measurements from ailing patients.[49] However, diagnosis is difficult due to its dependence on risk factors including, but not limited to; bacterial and viral respiratory lung infections,[50–52] alcohol abuse,[53,54] cigarette smoking,[55,56] and air pollution.[57,58] Unfortunately, only a minority of patients with these risk factors develop ARDS, leading to poor clinical recognition: 51% in mild ARDS and 79% in severe ARDS.[59,60] Another challenge in proper diagnosis is the need to quickly differentiate ARDS from mimicking syndromes including, but not limited to, acute eosinophilic pneumonia, diffuse alveolar hemorrhage, acute interstitial pneumonia, and acute heart failure.[49] Failure to differentiate these mimics from ARDS can contribute to the extremely rapid onset in disease progression. Therefore, effective therapeutics must not only mitigate symptoms of ARDS, but also treat the underlying risk factors.

Current treatment options for ARDS include mechanical ventilation, vasodilators, and corticosteroid administration.[10] These treatment options are limited in their effectiveness because they focus on reducing further injury, rather than addressing the underlying molecular mechanisms impacting patient survival. Human trials have shown that corticosteroids do not decrease ARDS frequency and do not improve patient outcome with administration at early disease stages.[61–64] Extracorporeal membrane oxygenation uses an external machine to pump and oxygenate a patient’s blood. This method in combination with carbon dioxide removal has not been shown to improve patient survival and actually results in substantial infection and bleeding risks.[65–68] Vasodilators, such as hydralazine and nitric oxide, have shown improved oxygenation and vascular resistance in ARDS patients; however, these improvements did not translate to

improved clinical outcomes.[69–71] While these interventions manage symptoms, ARDS is in need of new therapeutic strategies to reduce fatalities by treating underlying molecular mechanisms.

A promising therapeutic strategy is the use of noncoding RNAs to suppress expression of important genes involved in ARDS development and progression via RNAi. To date, there is only 1 FDA-approved RNAi-therapeutic; Patisiran, used to treat hereditary transthyretin-mediated amyloidosis.[72] In addition, there are multiple RNAi therapeutics in phase III clinical trials, including therapeutics to treat hemophilia,[73] hypercholesterolemia,[74] acute hepatic porphyria,[75] and primary hyperoxaluria type 1.[76] A recent phase I clinical trial also tested the efficiency of an RNAi-based proprotein convertase subtilisin–kexin type 9 for lowering low-density lipoprotein cholesterol; a 100 mg dosage reduced cholesterol levels by 50.6%, while a 300 mg dosage saw a 74.5% reduction in cholesterol.[77]

RNA interference. RNAi is a cellular mechanism that induces gene silencing.[78–85] The RNAi pathway is initiated when noncoding double-stranded RNA (dsRNA) is processed into single-stranded RNA.[86] There are 4 main categories of noncoding RNAs that participate in RNAi: long dsRNA and short-interfering RNA (siRNA), short hairpin RNA (shRNA), micro RNA (miRNA), and piwi-interacting RNA.

Exogenous dsRNA is processed into double-stranded siRNA by the endoribonuclease Dicer.[87] The siRNA-Dicer complex is then loaded into the RNA-induced silencing complex (RISC), where the double-stranded siRNA is unwound, the passenger strand is degraded, and the remaining guide strand is used for target mRNA recognition through complementary binding; strand selection is dictated by thermodynamic stability of the 5′ terminus of the guide strand.[83] (Fig 2) shRNA and miRNA are endogenous RNAs that operate by similar mechanisms. Both RNAs are transcribed in the nucleus, miRNAs by RNA polymerase II and shRNAs by either RNA polymerase II or RNA polymerase III, with their primary transcript containing similar stem-loop hairpin structures. The primary transcripts are processed by a ribonuclease III enzyme called Drosha and the heme-bound DGCR protein. The partially processed RNAs can then be transported to the cytoplasm by XPO5, where they are then cleaved and processed by Dicer and RISC in a similar manner as siRNA. Upon sequence recognition, the Argonaute-2 protein binds the mRNA to inhibit translational machinery or to signal for degradation, depending on siRNA-mRNA mismatching.[80,83] RNAi can also lead to heterochromatin formation, resulting in long-term or permanent gene silencing.[81,88]

Gene silencing via RNAi is a promising therapeutic strategy for treatment of ALI/ARDS that has the potential to modify the underlying molecular mechanisms of these disorders. The introduction of appropriate siRNA, shRNA, or miRNA to lung tissue in ALI/ARDS states can be utilized to prevent epithelial-endothelial dysfunction and lung cell death by targeting critical proteins for disease progression. An RNAi-based therapeutic strategy is conceptually simple and is limited only by accurate understanding of the roles played by various proteins in the disease pathway. In principle, delivery of siRNA to appropriate lung cells will result in mRNA degradation or translational suppression of the target gene(s) by the RISC.[87,89] In fact, there are multiple examples of cell-specific targeting of siRNA. siRNA has been exclusively delivered to dendritic cells in mice by decorating siRNA-containing immunoliposomes with dendritic cell-specific DEC-205 mAb.[90] Lymphocytes can also be targeted by using siRNA delivery vectors that can be recognized by RNAi-sensitive molecules such as retinoic acid-inducible gene-1 (RIG-1) and dsRNA-dependent protein kinase (PKR).[91,92] Similarly, lung endothelium has been targeted for cavelin-1 knockdown via IV injections of caveolin-1 siRNA/-cationic liposomes and the depletion of caveolin-1 by this approach resulted in increased lung vascular permeability in mice. These studies prove to be encouraging examples that can guide future research for developing novel delivery vectors to specifically bind the various lung cells involved in ARDS. While this strategy is conceptually simple, the therapeutic application of RNAi has been impeded by technical challenges that include delivery.[66,87,93–122]

The major obstacle for in vivo RNAi therapy is efficient siRNA delivery to the cell in a form that is accessible to the RNAi machinery. The most common type of cellular entry observed for macromolecules is clathrin-dependent endocytosis.[123] This results in the macromolecule being engulfed in an endosome, which then fuses with a lysosome, resulting in degradation of the endosome cargo.[123,124] Therefore, cellular uptake of therapeutic molecules via this mechanism requires a delivery system that enables early endosomal escape.[82,99–101,109,125] Oligonucleotides are poorly transported across the cell membrane and are subject to competitive degradation by endogenous nucleases. Therapeutic delivery of siRNA ideally requires a delivery mechanism that overcomes the poor cellular uptake of oligonucleotides and that stabilizes the siRNA to nucleolytic degradation prior to cellular uptake. In the next section, we will briefly outline common methods for drug delivery to the lung and the advantages and challenges of lung-specific siRNA delivery.

Pulmonary drug delivery. Pulmonary drug delivery is particularly amendable to tissue-specific delivery of

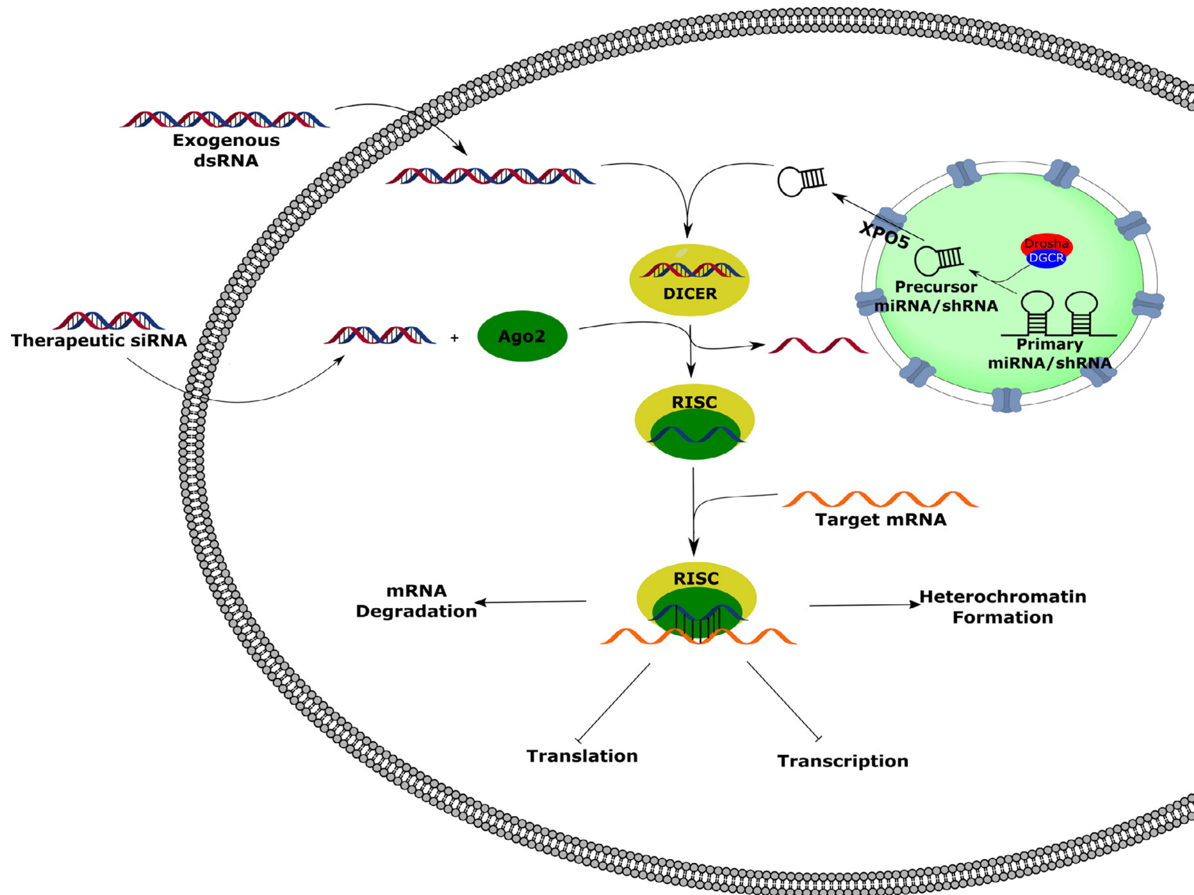

Fig 2. RNA Interference. Upon cellular entry, exogenous double-stranded RNA (dsRNA) is processed by Dicer and Ago2 to form the RNA-induced silencing complex (RISC). The guide strand directs the RISC to the targeted mRNA to silence gene expression by heterochromatin formation, mRNA degradation, or inhibition of transcription or translation. Endogenous miRNAs and shRNAs transcribed in the nucleus are processed by Drosha/DGCR prior to nuclear export by exportin-5 (XPO5). Once in the cytosol, the processed miRNA/shRNA can be incorporated into the RISC to induce genetic silencing. Therapeutic siRNAs introduced to the cell do not need to be processed by Dicer prior to forming the RISC. (Color version of figure is available online.)

siRNA. Drugs are commonly systemically administered either orally or intravenously, which requires that the therapeutic agent be sufficiently stable to survive circulation through various tissues and organs.[126] Nucleic acids are highly sensitive to degradation by serum nucleases in the bloodstream, making intravenous delivery problematic for siRNA therapeutics. [87,119,127] In contrast, there are advantages to developing an siRNA delivery method that targets the lung. The surface area of the human lung is approximately 50 times the area of the skin.[128] The human lung has approximately 480 million alveoli, with type I pneumocytes covering 95% of the alveolar space.[129,130] In total, the lung contains a vast capillary network spanning approximately 1000 km.[128] Due to the high volume of alveoli, the cells are typically in very close contact, eliminating the need for connective tissue between them; pneumocytes communicate across an alveolar pore measuring 8–60 μm.[130] Therefore, the architecture of the lungs independently confers a huge advantage for drug administration.

Although the lung architecture and vascularization provide significant advantages for drug delivery, the elaborate branched architecture of the lung also presents unique challenges.[87] There are 2 types of naturally present fluids in the airways that provide an additional barrier to pulmonary lung delivery: mucus and pulmonary surfactant. Mucus strongly interacts with drug molecules via electrostatic, hydrophilic, and hydrophobic interactions.[131] The continuous production and replacement of mucus in the airways efficiently traps and clears large particles.[132–134] Pulmonary surfactant produced by lung cells can strongly interact with nonviral cationic lipid-based delivery systems, impeding their effectiveness.[135,136] As a result, effective delivery of therapeutics to the

lung is heavily dependent on the mechanisms of particle deposition.[137] The ideal drug formulation particle size for pulmonary delivery is 1−5 μm because larger particles would be trapped in the upper airways, while smaller particles would be easily exhaled.[138,139]

The most commonly used methods for pulmonary drug administration are inhalation using metered dose inhalers (MDIs), dry powder inhalers (DPIs), or nebulizers.[115] MDIs are the most common inhalation method, requiring a pressurized formulation of the drug. In order to drive aerosolization of the drug it must be dissolved in a propellant, such as chlorofluorocarbons or hydrofluoroalkanes. DPIs function by administering the drug as a cloud of dry particles, however, it can be challenging to create a stable drug powder, making this method less common. Lastly, nebulizers are typically used for drug molecules that cannot be administered with MDIs or DPIs. Nebulizers function by generating a liquid aerosol which allows for large dosage administration.[115] Administration of a siRNA-based therapeutic would most likely use MDIs due to challenges that include formulation of biochemically stable siRNA-containing powders and an increased chance of siRNA degradation due to applied stress for DPI and nebulizers, respectively. An advantage of pulmonary delivery by these methods is that off-target effects observed by systemic delivery can be largely avoided since the bulk of the drug is retained in the lung.[115]

Unfortunately, treatment with mechanical ventilation for ARDS patients adds a unique challenge for delivery because drug particles cannot negotiate the ventilator circuits and endotracheal tubes.[140] MDIs require actuator devices for drug delivery to connect the inhaler with the pressurized circuits of the ventilators.[141] Drug delivery efficiency via this method varies with the design of the adaptor and the drugs/propellants used; in vitro studies have demonstrated extremely unpredictable efficiency ranging from 0.3% to 97.5%.[140,142−148] Nebulizer efficiency is also dependent on the type of nebulizer used and the positioning of the inhaler in the ventilation circuitry; inhalers with greater residual volume will afford a lower concentration of nebulized drug particles.[149,150] Dry-powder drug particle administration can engage the ventilator's inspiratory air flow to generate the aerosol or the drug particles can be introduced to the ventilator's air flow.[149]

SIRNA DELIVERY SYSTEMS AND THERAPEUTICS

The efficacy of potential siRNA therapeutics is highly dependent on the delivery system used. Naked siRNA shows low immune and inflammatory responses in vivo but is highly susceptible to degradation and has poor cellular transduction. Naked siRNA transduction occurs effectively only with the use of electroporation, sonoporation, jet injection, or related membrane disruption methods.[111] None of these methods have yet been tested in the lungs of patients, presenting an urgent need for development of improved methods for siRNA delivery. An ideal delivery system will be biodegradable, biocompatible, noncytotoxic, nonimmunogenic, and will adequately protect the payload from premature degradation.[114,119] In addition, the ideal delivery agent will facilitate translocation of siRNA cargo into cells without damaging the cell membrane or other cellular components and must bind siRNA reversibly to allow for selective release of the RNA from the carrier in the intracellular enviroment.[114,119] The different vectors that have been utilized for siRNA therapeutics are briefly discussed below, with their general pros and cons outlined in Table 1.

Viral vectors. ***Adenovirus.*** Adenovirus is the most widely used viral vector in lung-targeted oligonucleotide therapeutics.[111] For the virus to reach its receptor on the basolateral surface of lung epithelial cells in an ALI/ARDS lung, adenovirus must rely on transient barrier dysfunction.[155] Clinical trials and animal models have shown that use of adenovirus clears transduced cells and limits the number of tolerated doses.[151,163] Adenovirus has been demonstrated to be efficient in the delivery of noncoding RNA in lung cancer and sepsis-induced lung injury. Adenovirus expressing matrix metalloproteinases-2 siRNA was shown to mitigate metastasis and caner-induced angiogenesis.[164] The model studied consisted of A549 human lung carcinoma cells in severe combined immunodeficient mice. Treatment with adenovirus-expressing metalloproteinases-2 siRNA resulted in a 60% reduction in tumor volume and no metastatic foci in mouse lung tissues.[164] C5aR-shRNA was delivered via an adenovirus vector in sepsis-induced mice.[165] Significant knockdown of C5aR, which has been shown to be important in lung permeability, was achieved 4 days postadministration.[165,166]

Adeno-associated virus. Adeno-associated virus (AAV) is a nonpathogenic vector that overcomes some of the deficiencies encountered with adenovirus as a vector for oligonucleotide delivery.[155,167] The AAV vector genomes are approximately 5 kilobases in length, greatly limiting the size of the gene encoding the noncoding RNA.[154] siRNA has been successfully delivered to HeLa S3 cells via an AAV vector to silence p53 and caspase-8 expression.[168] shRNA expressing AAV was used to target eotaxin-1 in an in vivo study conducted in mice to treat chronic asthma.[169] Intratracheal injection of the AAV vector reduced levels of IL-4, IL-5, and IL-

Table 1. Summary of the advantages and disadvantages of different viral vectors that have been utilized for siRNA delivery

Delivery vector		Advantages	Disadvantages
Viruses	Adenovirus	Highly efficient transduction profile[111]	Immunological responses, nonspecific cell targetting,[111] acute toxicity[151]
	AAV	Highly efficient transduction profile,[111] reduce inflammatory and immune responses[152,153]	Requires helper virus,[152] small cloning capacity[154]
	Retrovirus	High gene transduction	Dependence of cell differentiated state,[155] insertion into host genomes[115]
	Lentivirus	High gene transduction, cell differentiated state independence[156]	Insertion into host genomes[115]
Lipids	Cationic	Good transfection efficiency, electrostatic interactions with siRNA[115]	Poor stability, cellular toxicity, immune response elicitation[106,157]
	Neutral	Reduced cytotoxicity[115]	Reduced ability to complex with siRNA[115]
	Nanoparticles	Charge variable, complex formation prior to administration, neutralized at physiological pH, reduced toxicity and immunological responses, increased membrane destabilizing capacity, higher endosomolytic activity[110,115,158]	Synthetic challenges
Polymers	Polycations	Modifiable size[115]	Weaker interactions with siRNA[115]
	Nanoparticles	Biocompatible, biodegradable, synthetic diversity[115]	Large surface area causes aggregation[159]
Peptides	PLL	Electrostatic interactions with siRNA, synthetic diversity[120]	Toxicity, nonspecific binding, poor endosomal escape[102,160,161]
	CPPs	Synthetic diversity, covalent and noncovalent complex formation[120]	Membrane permeability challenges, poor endosomal escape[120,162]

10 in the BAL fluid and reduced polymorphonuclear leukocyte infiltration.

Retroviruses. Retroviruses have also been utilized for drug delivery to the lung. In a cystic fibrosis study, a replication-deficient CFTR HIV viral vector demonstrated high gene transduction in a differentiated cystic fibrosis-derived human bronchial cell xenograph, while a fully functional CFTR HIV vector could not.[170] In another study, a lentivirus was used to deliver p65-shRNA in mouse lungs, resulting in inhibition of NF-κB activity.[171] Lentiviral livin-shRNA was delivered to lung adenocarcinoma xenografts in mice; gene silencing resulted in tumor cell apoptosis and reduced cellular proliferation and growth[172] (Fig 3).

Nonviral vectors: lipids. ***Cationic lipids.*** Cationic lipids can spontaneously form lipoplexes with negatively charged siRNA.[115] A recent study tested 6 cationic cholesterol derivatives, 11 cationic glycerol-based derivatives, and 17 cationic liposomes to compare the efficiency of Tie2 siRNA delivery to lung tissue when injected intravenously in mice.[173] The cholesterol derivates demonstrated very efficient gene silencing, while glycerol-derivatives with short-length alkyl chains and long linker arms decreased the efficiency of gene silencing. However, accumulation was not consistent, with some complexes accumulating in the liver. The authors also demonstrated that 3 glycerol-based derivatives could suppress tumor growth in mice with Lewis lung carcinoma (Fig 4).

Neutral lipids. Neutral lipids are significantly less cytotoxic than cationic lipids.[115] However, the loss of net charge significantly reduces their ability to effectively complex with negatively charged siRNA.[115] Methods for chemically conjugating cholesterol-derived lipids to siRNA have been developed to overcome this disadvantage.[82,125] This conjugation reduced the inflammatory response and knockdown duration but did not improve knockdown.[125] Cationic lipids modified with neutral polymer polyethylene glycol have been studied for siRNA delivery with the aim of reducing immunological responses and cellular toxicity.[115] Genzyme lipid (GL-67) is an example of a neutral polyethylene glycolylated lipid that has been successfully utilized for plasmid DNA and siRNA delivery to lung cells.[174–176] (Fig 5) β-galactosidase-siRNA was delivered to mouse lung via intranasal administration, resulting in 33% knockdown in alveolar cells.[176]

Lipid nanoparticles. Ionizable, pH-sensitive amino lipids have been developed to encapsulate oligonucleotides in the form of lipid nanoparticles.[158] The method was improved by developing stable nucleic acid lipid particles with 2 distinctive properties: the ability to induce nonbilayer phase structure with anionic lipids and the ability to tune the surface charge by modifying the pK_a of the lipid constituents.[110]

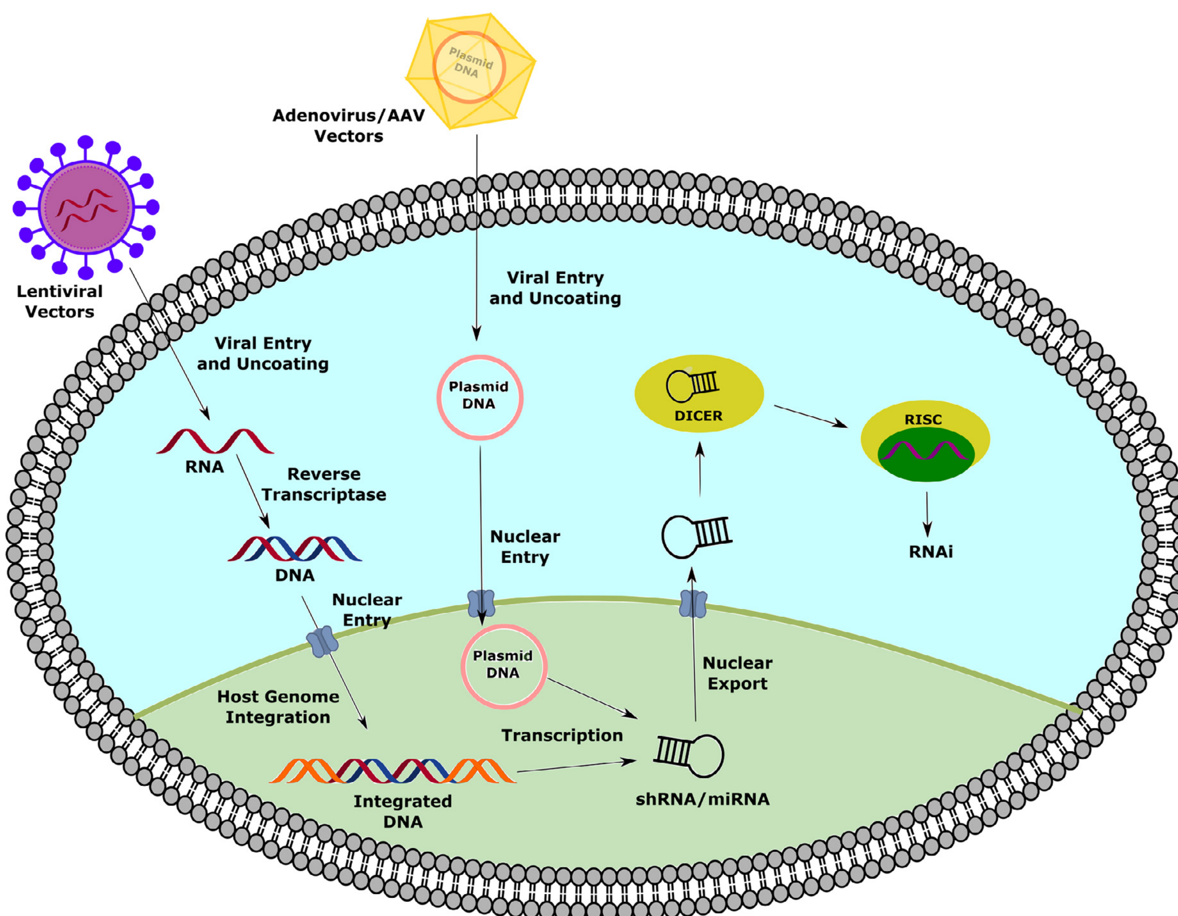

Fig 3. Delivery of noncoding RNAs with different viral vectors. Viral vectors act by different mechanisms to produce siRNAs. Lentiviral vectors are uncoated upon cellular entry, which releases their single-stranded RNA (ssRNA) into the cytosol. Reverse transcriptase encoded by the virus allows the ssRNA to be transcribed to double-stranded DNA (dsDNA), which can integrate in the host genome upon nuclear entry. Adenovirus/AAV vectors carry a DNA plasmid encoding for the siRNA, which doesn't require extensive processing prior to nuclear entry. Both viral vectors use the host genome to transcribe the encoded siRNA, which can then be exported from the nucleus to enact gene silencing. (Color version of figure is available online.)

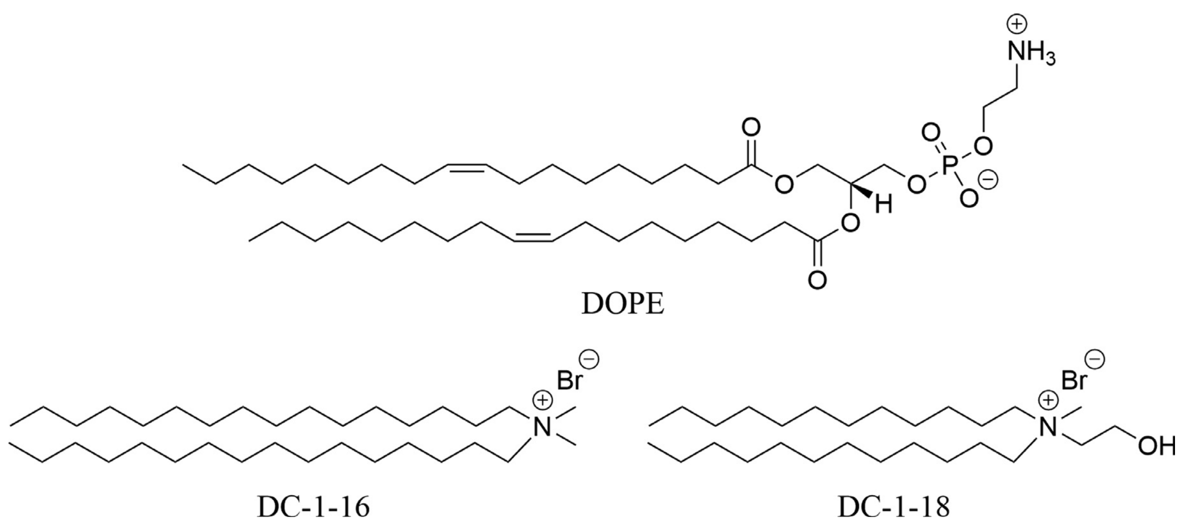

Fig 4. Examples of cationic lipids used for non-coding RNA delivery. DC-1-16 and DC-1-18 were used in conjugation with DOPE to treat Lewis lung cell carcinoma by suppressing tumor growth in mice.

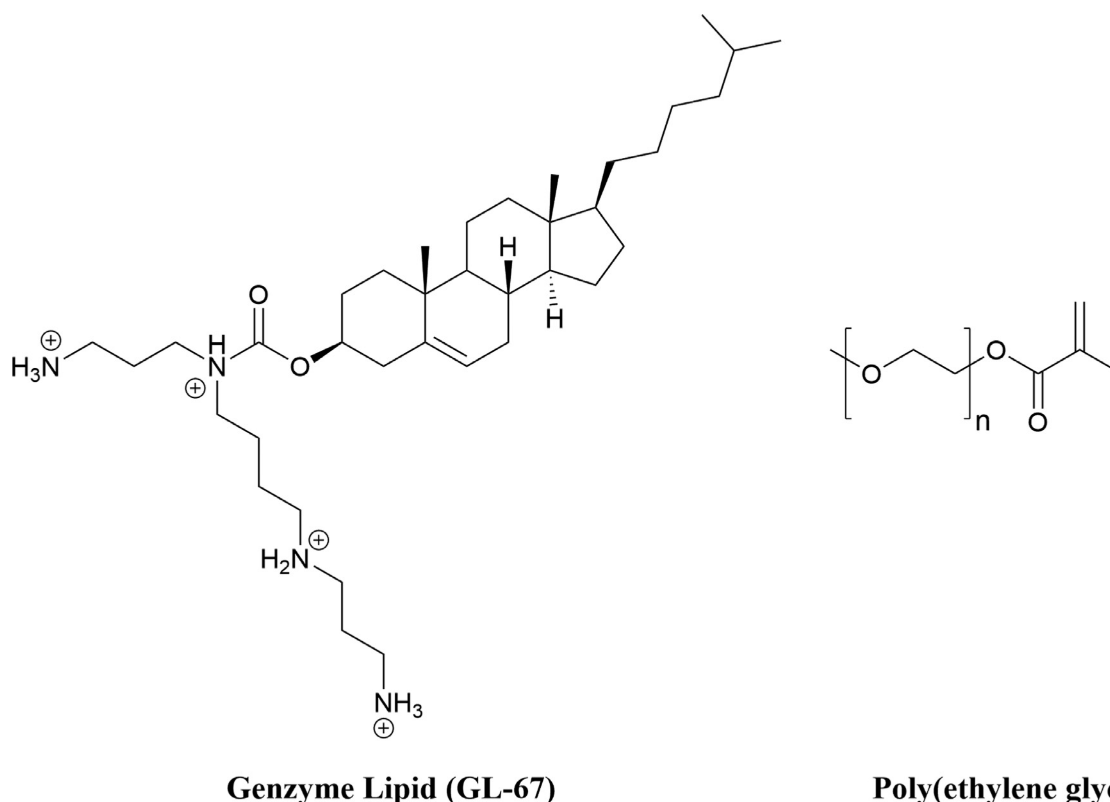

Fig 5. Chemical structures of GL-67 and PEG. Poly(ethylene glycol) has been utilized with many different delivery vectors to enhance cellular uptake One such example, is the PEGylated Genzyme Lipid that was used to deliver antisense oligonucleotides and siRNA to mice lung epithelia.

Nonviral vectors: polymers. Polymer-based delivery systems have also been explored for siRNA delivery. Polycations and polymeric nanoparticles are two polymer types which have been utilized for siRNA delivery.[115]

Polycations. Polycations with a high charge density form electrostatic complexes with negatively charged siRNA.[115] The size of polymer-siRNA polyplexes can be modified as a function of polymer molecular weight, polymer charge ratio, solution pH, and solution ionic strength. Although delivery with DNA has been highly successful, siRNA has proven more difficult due to the stiffer RNA structure, resulting in weaker interactions, making the siRNA more susceptible to degradation.[115] Polyethylenimine (PEI) has demonstrated high intracellular efficiency for siRNA delivery. The high buffer capacity of PEI results in swelling of the complex in the endosome environment, which results in subsequent rupturing of endosomes. Unfortunately, the high toxicity and nonbiodegradability of PEI has limited its application as an effective delivery system.[177] Chitosan is a natural cationic polysaccharide polymer that has advantages over PEI for siRNA delivery due to its biocompatibility, biodegradability, decreased cytotoxicity, and mucosal permeation properties.[107,178,179] (Fig 6) Transgenic endogenous green fluorescent protein (EGFP) mice were used to study the efficiency of the pulmonary delivery by a chitosan-based siRNA vector.[178] Intratracheal administration of chitosan-siRNA nanoparticles over a 5-day period demonstrated 43% knockdown of EGFP in bronchial epithelial cells.[178] A more recent study used a nebulizing catheter for chitosan-siRNA delivery in transgenic EGFP mice, resulting in an improved 68% knockdown of EGFP in mice lungs.[180]

Polymeric nanoparticles. Polymeric nanoparticles are primarily formed from hydrophobic polymers and act to encapsulate siRNA in the surrounding polymer matrix.[115] Poly(D,L-lactic-co-glycolic acid) (PLGA) is a copolymer that has been used for gene therapy in the lung.[115] PLGA is a copolymer derived from lactic and glycolic acid. Poly glycolic acid is not utilized because it is more rapidly degraded via hydrolysis in the body.[181] Addition of lactic acid motifs with methyl side groups, increases the hydrophobicity of the polymer, decreasing the degradation rate of the polymer.[181] (Fig 7) Many derivatives of this copolymer can be developed with tailored properties. For example,

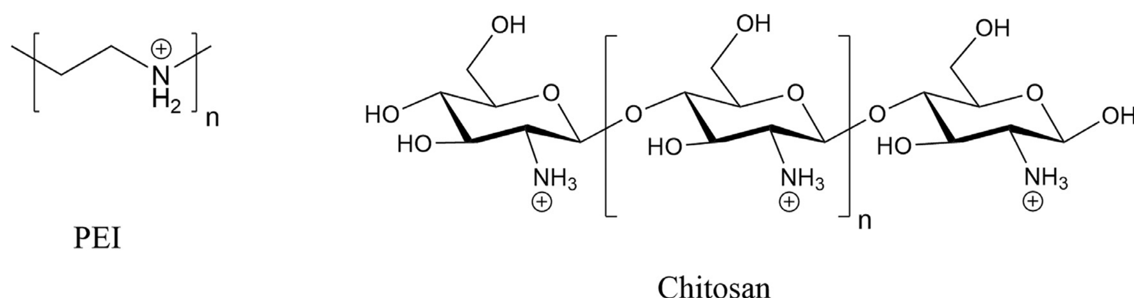

Fig 6. Chemical structures of PEI and Chitosan. Polyethylenimine (PEI) has been used in conjugation with other delivery systems to enhance cellular delivery of siRNA. Chitosan, a linear polysaccharide, has been used as a biocompatible and biodegradable alternative to PEI.

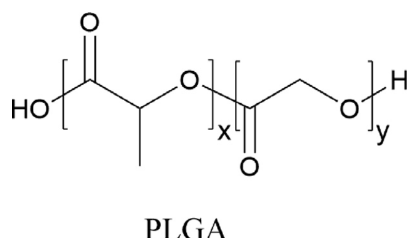

Fig 7. Chemical structure of PLGA copolymer. PLGA is a copolymer derived from lactic and glycolic acid for nucleic acid delivery. Hydrolysis in the body to yield lactic and glycolic acid greatly reduces its toxicity. By altering the number of monomers incorporated into PLGA, the degradation rate of the copolymer can be altered to alter payload release rates.

sustained payload release can be obtained by altering the block copolymer composition and molecular weight, which changes the degradation rate of the polymer.[182]

Nonviral vectors: peptides. *Poly(L-lysine) (PLL).* Poly (L-lysine) (PLL) is a polymer of the amino acid lysine that can interact with siRNA via electrostatic interactions between the negative phosphate backbone and positive lysine residues.[120] (Fig 8) PLL is a versatile carrier as complex formation can be influenced by polymer molecular weight, pH, charge ratio, salt concentration, and mixing order.[183]

Cell-penetrating peptides (CPPs). A wide range of cell-penetrating peptides (CPPs) have been exploited for siRNA delivery. Some of the most commonly used CPPs for siRNA delivery include transportan,[184] VP22,[185] MAP,[186] and synthetic arginine-rich peptides.[187,188] Cellular uptake of siRNA complexed with CPPs can occur via endocytic or nonendocytic pathways, depending on the particle size, peptide type, and siRNA loading method.[120,162] Covalent peptide complexes ensure that the conjugate remains intact, however, this may reduce the ability of the peptide to penetrate the cell membrane by neutralizing important side chains.[120] Proteins and peptides containing dsRNA binding domains have also been complexed with siRNA for cellular delivery.[120] HIV-1 *trans*-activator protein (TAT) has been used for delivery of p38 MAP kinase siRNA to the mouse lung via intratracheal administration.[125] (Fig 8) Disulfide-linked conjugation of the siRNA with TAT(48−60) resulted in 20%−30% knockdown 6 hours after administration. TAT is a commonly used CPP, however, some studies have shown nonspecific cellular uptake with this vector.[84] Other CPPS that can be used in siRNA delivery include: Protamine 1,[189] Penetratin-1[190] $(PPR)_n$,[191] $(PRR)_n$,[191] DPV peptides,[192] MPG,[193] POA,[194] viral proteins,[188,195,196] and others.[197-213]

Recently, our lab has developed a cyclic amphipathic peptide (CAP) delivery system for siRNA delivery to the lung.[122] Our peptides consist of alternating hydrophobic and hydrophilic amino acids flanked by a cysteine residue at each terminus. The peptides are cyclized by oxidative intramolecular disulfide bond formation between the thiol groups of these flanking cysteines. (Fig 8) These peptides form noncovalent complexes with siRNA that effectively transport siRNA into the cytosol by a mechanism that we are currently working to elucidate. The cyclic peptides are resistant to degradation by proteases and protect the complexed siRNA from degradation by nucleases. Upon cellular entry, the peptide disulfide bonds are reduced in the cytosol and the linearized peptides are proteolytically degraded, resulting in release of the siRNA payload. Cyclic Ac-C$(FKFE)_2$CG-NH_2 and Ac-C$(WK)_4$CG-NH_2 peptides complexed with thyroid transcription factor-1 siRNA to form CAP-siRNA nanoparticles that were tested *in vitro* in human A549 lung epithelial cells and *in vivo* in mouse lung to show 80% knockdown of transcription factor-1 in each. These particles were delivered to mouse lung by aspiration. We are currently applying our CAPs for delivery

TAT (48-60)

Poly(L-lysine)

$C(WR)_4CG$

Fig 8. Chemical structures of cell-penetrating peptides to deliver noncoding RNAs. HIV-1 TAT (48-60), cyclic $C(WK)_4CG$, and poly(L-lysine) have been used for nucleic acid delivery. These peptides are cationic at physiological conditions, forming strong electrostatic interactions with nucleic acids. The structure of these peptides inhibits passive diffusion across the cell membrane, requiring an endocytosis-mediated mechanism for payload delivery.

of ALI-relevant siRNA to explore this as a strategy to intervene in the development of ALI.

POTENTIAL ALI/ARDS TARGETS FOR RNAI INTERVENTION

With recent advancements for pulmonary delivery of siRNA, RNAi strategies have great potential as interventions for treatment of ARDS. The realization of this potential relies not only on the development of effective delivery modalities, but also on identification of appropriate ARDS gene targets for RNAi knockdown. Potential therapeutic targets for the treatment of ARDS include proteins affecting epithelial-endothelial dysfunction, neutrophil infiltration, and cell death. (Table 2) Much work has been done to find prospective siRNA targets for the modification of lung pathology and to reduce the mortality rate of ARDS. None of these potential targets are yet the subject of clinical trials as therapeutics, but current research is encouraging. In the remaining sections we will outline promising therapeutic targets for RNAi interventions and briefly discuss the data that illustrates the potential of these targets.

Therapeutic targets for reducing vascular permeability. The claudin family of proteins is important for forming intercellular barriers at tight junctions.[214] Claudin-4, specifically, is known to limit paracellular sodium transport and create chlorine-selective paracellular pores.[215] Wray et al have used RNAi to demonstrate that claudin-4 knockdown resulted in leakier tight junctions at the endothelial-epithelial barrier, which results in decreased alveolar fluid clearance and increased pulmonary edema.[214] In fact, claudin-4 expression is

Table 2. Summary of the potential therapeutic targets and their impact on the cellular signaling pathways in ARDS

Target	Promotes	Inhibits	Epithelial apoptosis	Lung edema	Alveolar leakage	Barrier integrity	Inflammation	Inflamm. cell infiltration	Inflamm. mediators
Rip2	NF-κB	-	-	P	P	I	P	P	-
RPS3	NF-κB	-	-	P	P	I	P	P	-
NF-κB	KC, IL-17, Rip2	-	-	-	-	I	P	P	-
Caspase-3	KC, MIP-2	-	P	P	P	-	P	P	-
S1P	IL-6, TNF-α	-	-	-	-	-	P	P	-
IL-17	-	-	-	-	-	-	-	P	-
Fas	Caspase-3, TNF-α, IL-6, IL-12	-	P	-	-	-	P	P	-
IL-6	-	IL-10	-	P	P	-	P	P	-
MTOR	-	NF-κB	-	-	-	P	I	I	P
KC	IL-6	-	-	P	P	-	P	P	-
MIP-2	-	-	-	-	-	-	P	-	-
IL-12	-	-	-	-	-	-	P	-	-
IL-10	-	-	-	I	I	-	I	-	-
TNF-α	-	-	-	-	-	-	P	-	-

Abbreviations: I, inhibits; P, promotes.

decreased in early stage ventilator-induced ARDS.[214] Based on this data, claudin-4 itself is obviously not an appropriate target for ARDS RNAi intervention. However, additional research that identifies the upstream effectors of claudin-4 downregulation in ARDS progression may present novel valid targets for siRNA knockdown maintaining healthy pulmonary vascular permeability.

Epithelial-endothelial barrier dysfunction is also impacted by angiopoietin-2 (Ang-2).[216] Ang-2 is a proinflammatory antagonist for the Tie-2 receptor found in high concentrations on lung endothelial cells, which acts by inhibiting the antiinflammatory angiopoietin-1 ligand from phosphorylating Tie-2.[217] Inhibition of this phosphorylation also results in the upregulation of phosphorylated MLC, which increases endothelial barrier permeability.[218,219] A small clinical study found an increase in circulating Ang-2 levels in ARDS patients.[216] Exposure of cultured endothelial cells to plasma from ARDS patients with high Ang-2 levels caused disruption of the endothelial barrier and this response was attenuated in the presence of angiopoietin-1, supporting the molecular mechanism of the role of Ang-2 in barrier dysfunction.[216] Lung-specific down-regulation of Ang-2 via RNAi is thus a potential therapeutic target to improve the clinical outcomes of ARDS patients.

Therapeutic targets for reducing inflammation and cell death. Keratinocyte-derived chemokine (KC) and macrophage-inflammatory protein-2 (MIP-2) are 2 neutrophil-attracting chemokines that share a common affinity for CXC chemokine receptor 2.[220] Upregulation of KC and MIP-2 was observed in a mouse model of ARDS.[221−223] Naked KC and MIP-2 siRNA were administered via intratracheal administration 2 hours after inducing ARDS in order to assess the effects of downregulation of these proteins.[221] Local MIP-2 levels were not reduced by KC nor MIP-2 silencing, indicating that circulating MIP-2 is more likely to be associated with the lung's inflammatory environment during ARDS. Decreased levels of interleukin-6 (IL-6) was 2 times lower with KC than with MIP-2 silencing. This study demonstrated a 40% suppression of chemokine expression. Based on this data, knockdown of KC in the lung may have therapeutic value for ARDS treatment, while systemic MIP-2 silencing, rather than lung-specific MIP-2 knockdown, may be necessary to modify ARDS.

The effects of direct caspase-3 knockdown have also been interrogated.[40] The effects of 4 different caspase-3-siRNA duplexes were compared following intratracheal administration of these siRNAs in mice following hemorrhagic shock. Transfection of caspase-3-siRNA resulted in over 90% knockdown of caspase-3 mRNA,

protecting epithelial cells from caspase-dependent apoptosis.[40] Caspase-3 knockdown suppressed TNF-α, IL-6, and KC levels within 24 hours, resulting in a reduction in neutrophil infiltration. Systematic inflammation was reduced following a decrease in local and plasma TNF-α, IL-6, KC, and MIP-2 levels. The suppression of caspase-3 also resulted in improved alveolar architecture and reduced alveolar leakage. Mice that received the caspase-3-siRNA had a 10-day greater survival rate than the control group, indicating the promise of caspase-3 as an RNAi target for potential ARDS intervention.[40]

IL-6 is also a potential target for siRNA-based therapies. A lentiviral vector was recently used to deliver IL-6 shRNA to rat lung following intestinal ischemia-reperfusion (II/R).[224] Intestinal II/R involves reoxygenation of the intestines following vascular blockage for a prolonged period of time.[225] This type of injury activates the immune system due to the production of ROS, cytokines, and chemokines, leading to distal organ dysfunction, particularly in the lung.[224,225] II/R significantly increased IL-6 expression, with the highest concentration recorded 8 hours postinjury. The shRNA-lentivirus complex was delivered by direct injection to lung tissue. IL-6 levels and red blood cell concentration was significantly decreased in pulmonary alveoli 16 hours postinjury. Silencing of IL-6 increased IL-10 expression and reduced lung edema and alveolar leakage, indicating that IL-6 is a candidate for RNAi intervention in ARDS.

Homeodomain-interacting protein kinase 1 (HIPK1) is an important pro-oncogene involved in regulating apoptosis and DNA-damage repair.[226–228] Lipopolysaccharide-induced ARDS mice exhibited elevated levels of HIPK1. Lipofectamine-delivered HIPK1 siRNA significantly reduced HIPK1 mRNA expression and reduced IL-6 and TNF-α levels. This indicates that HIPK1 may also be an appropriate ARDS target for RNAi intervention.

Another potential target is sphingosine-1-phosphate (S1P), which reduces acute pulmonary inflammation by blocking NF-κB signaling in macrophages.[229] Inhibition of NF-κB impacts barrier integrity, cytokine release, and neutrophil infiltration.[229] S1P lyase (S1Plyase) is an essential enzyme for S1P activity; silencing of this enzyme inhibits S1P, thereby, reducing inflammation and neutrophil infiltration.[229] Oh and Lee silenced S1Plyase activity by intratracheally coadministering S1Plyase siRNA with high mobility group box-1 peptide (HMGB1A). High mobility group box-1 (HMGB1) induces ARDS by releasing inflammatory cytokines; HMGB1A functions as an antagonist of HMGB1.[230–232] Nanoparticles were formed by using R3V6 peptide as an S1Plyase siRNA carrier and administration of these particles reduced S1Plyase, IL-6, and TNF-α concentrations in BAL fluid. The authors also compared siRNA delivery by these nanoparticles to delivery with PEI and Lipofectamine carriers. The R3V6 peptide demonstrated higher delivery efficiency and was found to be less cytotoxic than the PEI and Lipofectamine nanoparticles. HMGB1A has also been linked to lung-epithelial binding peptide (LEBP).[233] LEBP linkage resulted in epithelial-specific transfection, with an improved transfection efficiency over PLL or the non-LEBP HMGB1A conjugate. The use of this linker was shown to be noncytotoxic.

Receptor-interacting protein 2 (Rip2) positively regulates the NF-κB pathway and is upregulated by NF-κB expression.[234] Patients in late stage ARDS demonstrate significant upregulation of Rip2, making it an important potential target for silencing.[235] Lipofectamine-delivered Rip2 siRNA was administered intratracheally to mice for 3 consecutive days following extensive cigarette smoke (CS) exposure for 5 weeks.[236] Rip2 knockdown reduced neutrophil and lymphocyte infiltration, suppressed proinflammatory mediators in the BAL fluid, and inhibited expression of TNF-α and KC, indicating the potential of Rip2 as a target for RNAi intervention.

NF-κB can also be directly targeted to modify ARDS progression. Lipofectamine was used to deliver NF-κB siRNA to sepsis-induced ARDS in mice. Sepsis induced by cecal ligation and puncture was observed 3 hours after surgery.[237] IL-17 and IL-6 count increased 20-fold in the BAL fluid 6 hours and 12 hours postsurgery, respectively. Administration of NF-κB siRNA reduced inflammatory cell infiltration and TNF-α expression after 2 hours. Lung histology was improved and IL-6 and IL-17 levels decreased 12 hours after treatment. However, IL-6 and IL-17 levels reached their minimum at 6 hours.[237]

Another study targeted NF-κB by targeting ribosomal protein S3 (RPS3) in a CS induced ARDS model.[238–240] RPS3 is an integral part of NF-κB, and is therefore required for correct function. CS exposure increased macrophage, neutrophil, IL-6, KC, and TNF-α levels in the BAL fluid. Administration of RPS3 siRNA reduced levels of all proinflammatory factors and neutrophils. CS exposure correlated with an increase in epithelial wall thickness; RPS3 siRNA knockdown reduced this thickening. The NF-κB p65 subunit and RPS3 nuclear concentrations were both reduced following siRNA treatment.[238]

Recently, it has been reported that mechanistic target of rapamycin (MTOR) has a complex involvement in ARDS pathology.[241,242] While some studies have shown that MTOR promotes lung inflammation and injury, others have reported an inhibitory role of MTOR in inflammatory lung injury.[241–243] The differential effects

of MTOR signaling on ARDS can be ascribed to a cell-specific role of MTOR. We and others have shown that it functions as an anti-inflammatory molecule to limit endothelial cell inflammation, whereas it serves a proinflammatory function to promote inflammation in epithelial cells.[243,244] Similar proinflammatory and anti-inflammatory roles for MTOR have also been reported in neutrophils and monocytes/macrophages, respectively.[242,245] This data demonstrates a significant need for therapeutics with high cell specificity, since it appears that downregulation of MTOR in the lung epithelium and coincident upregulation in the lung endothelium is optimal for ARDS intervention. We have initiated studies to exploit disulfide-constrained CAPs in complex with MTOR siRNA to understand the selectivity of MTOR knockdown in epithelial and endothelial cells in the lung. The etiology of ARDS is increasingly understood to be highly nuanced, and greater understanding of disease-modifying proteins is necessary to validate efficacious protein targets for siRNA-based interventions.

Therapeutics for Active inflammation resolution and pulmonary repair. Although prevention of ARDS progression has been the focus of therapeutic development, therapeutics that activate beneficial innate immunity pathways would also serve to benefit ARDS patients. Regulatory T-cell activation and clearance of apoptotic/necrotic cells by phagocytic cells has been shown to be important for lung repair in ARDS patients.[246,247] One way in which T-cells promote active repair is by secreting IL-10, suggesting that upregulation of T-cell activation by RNAi can be a useful therapeutic tool.[247–249] Regulatory T-cell activation and differentiation is dependent on CD4 T cell coreceptor, CD25,[250,251] and Forkhead box P3 (FoxP3)[252], making these additional targets for RNAi therapy.

CONCLUSIONS

ARDS is a serious medical condition with a very high mortality rate, due to difficulty in adequately diagnosing the disease. Current methods of treatment focus on preventing further tissue damage without addressing the underlying mechanisms involved. RNAi is an attractive choice for therapeutic development because it can both prevent further pulmonary damage and reverse current pulmonary damage before serious disease progression occurs. Although there are many advantages to pulmonary delivery, there are multiple lung-specific barriers that need to be overcome. In addition, siRNA requires a delivery system which is biodegradable, biocompatible, noncytotoxic, nonimmunogenic, and that adequately protects the payload from degradation and binds siRNA reversibly to allow for efficient release of the drug. Although different viral and nonviral vectors have been developed, none are ideal and are in need of further development. While the architecture of the lung and the possibility for isolated delivery to the lung is advantageous for selective siRNA delivery, challenges presented by mucous and pulmonary surfactant A need to be overcome when designing effective delivery vectors. Potential targets for the treatment of ARDS are extensive, with new targets still being discovered, making RNAi a promising therapeutic strategy. Overcoming significant deficits with understanding of ARDS progression as well as the development of next-generation delivery vectors are both necessary in order to realize the potential of RNAi intervention as a treatment modality for ARDS.

ACKNOWLEDGMENTS

Declaration of competing interest: All the authors have read the journal's policy regarding disclosure of potential conflicts of interests and declare that there are no conflicts of interest.

Preparation of this manuscript was supported by grants from the National Institutes of Health, National Heart, Lung, and Blood Institute (R01HL138538 and R01HL120521).

Author contribution: The journal's authorship agreement has been reviewed and the manuscript has been reviewed and approved by all authors involved.

We also thank Jade Welch for editorial suggestions during the preparation of the manuscript.

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