miR-Based Treatments for Acute Respiratory Distress Syndrome

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Acute Respiratory Distress Syndrome (ARDS) is characterized by lung inflammation and increased membrane permeability, which represents the leading cause of mortality in ICUs. Mechanical ventilation strategies are at the forefront of supportive approaches for ARDS. Recently, an increasing understanding of RNA biology, function, and regulation, as well as the success of RNA vaccines, has spurred enthusiasm for the emergence of novel RNA-based therapeutics. The most common types of RNA seen in development are silencing (si)RNAs, antisense oligonucleotide therapy (ASO), and messenger (m)RNAs that collectively account for 80% of the RNA therapeutics pipeline. These three RNA platforms are the most mature, with approved products and demonstrated commercial success.

Keywords: ARDS ; gene therapy ; miRNA ; nanotechnology

1. Introduction

There are over 60 known causes of Acute Respiratory Distress Syndrome (ARDS) [1][2], including pneumonia (e.g., infection by bacteria [3][4], viruses [5][6][7][8], and fungi [9]), systemic diseases (e.g., sepsis) [10][11], severe trauma [12], multiple transfusions [13], chemotherapy [14], toxic inhalations [15], pancreatitis [16], and chemical/biological warfare [17]—all of which may require advanced respiratory support such as supplemental oxygen, mechanical ventilation (MV), or extracorporeal membrane oxygenation (ECMO) [18].

Irrespective of the cause, all pathways of acute lung injury (ALI)—the biological process underlying ARDS—converge on shared histological and molecular features, including flooding of the lung airspaces with edema fluid [19], activation of immune cells and secretion of inflammatory mediators causing immune dysregulation [20], destruction of lung tissue, and degradation of surfactant. Increased dead space (perfusion of non-aerated alveoli) and decreased pulmonary compliance (stiffening of the lungs) result in impaired gas exchange; thus, the need for respiratory mechanical support [20][21][22]. When injury in the lung reaches a critical threshold, inflammation spills over into the circulation (loss of pulmonary compartmentability), to affect other organs, leading to multi-organ failure (such as heart, kidney, brain, and liver failure) and ultimately death [23].

In this new era of genomic medicine, RNA-based therapies are particularly attractive, especially when transient regulation of gene expression profiles may be enough to redirect innate immune responses without permanently altering the genetic makeup of the cell. MicroRNAs (miRs) are small RNAs with a length of 20–24 nucleotides that control gene expression post-transcriptionally [24][25][26]. miRs work as gene silencers by complementary base-pairing to the 3′ untranslated regions (UTRs) of mRNAs [25][27][28]; miRs can then destabilize and degrade the target mRNA or suppress its translation [29][30]. The requirement for base-pairing by way of nucleotides at positions 2–8 of the mature miRNA termed the “seed sequence” makes them highly specific [31]. Herein lies the attraction of miRs as basic therapeutic agents: delivery of miR mimics or inhibitors can be used to target therapeutically relevant miRs [32][33].

2. Identifying Therapeutically Relevant microRNAs

miRs seem to be involved in all cellular processes; while detecting differentially expressed miRs associated with particular conditions, distinguishing causally and therapeutically relevant miRNAs from epiphenomenal changes is challenging. The approach taken by various groups, is to utilize advanced algorithms to effectively analyze large and complex networks of relationships between biomedical entities, and to embrace data-driven decision-making in target assessment and prioritization to promote innovation by using digital discovery platforms to generate innovative hypotheses about potential therapeutic targets that can be tested in vitro and in vivo.

The discovery approach relies upon system perturbation and identification of attractor states in high dimensional data analysis [34]. In the case of the ARDS studies, authors perturbed the system by treating septic mice with mesenchymal stromal cells (MSCs) [35][36][37][38]. MSCs have been shown to effectively prevent lung injury and organ dysfunction by reducing inflammation and enhancing bacterial clearance [37][39][40][41][42]. Authors investigated the transcriptome and microRNAome of cases versus controls treated with MSCs versus placebo to identify mRNA:miR pairs regulated in both cases and controls, as well as placebo vs. MSC in five major organs affected by sepsis (lungs, liver, kidney, spleen, and heart). Authors combined gene set enrichment analysis with co-regulation of mRNAs and miRs to identify possible therapeutic targets on the assumption that, while one regulated miR may be interesting, cumulative co-regulation of an miR (or a group of related miRs) and its known putative target (or group of targets) in all conditions (e.g., sepsis) across more than one organ denoted a highly causal relationship with the condition or trait of interest, allowing us to filter out epiphenomenal changes [35]. Candidate mRNA:miR pairs that emerged from this analysis were ranked and prioritized for future empiric validation using strict criteria, including: (i) statistical significance of changes (standard deviation amongst replicates), (ii) magnitude of change (effect size), (iii) sequence homology between mice and humans (to facilitate future translation to humans), (iv) pathways enrichment that related to disease of interest (biological plausibility), and (v) the ability to experimentally test a novel in silico hypothesis—to measure and evaluate an effect on putative biotargets and function in vitro and in vivo.

Importantly, identified biomarkers may be associated with but not causatively related to the disease pathophysiology. For instance, Zhu et al. used miR profiles and logistic regression analyses to identify the miR markers associated with ARDS [43]. However, this does not imply a cause-effect relationship between the dysregulation of the miRNA and disease pathogenesis. Experimental validation is fundamental to verifying in silico predicted relationships and biological effects.

In linking specific miRs with ‘treatable traits’, García-Hidalgo et al. utilized statistical models to investigate the relationship between miRs and pulmonary function, providing insight into the potential molecular pathways for SARS-CoV-2-induced ARDS [44]. Lung diffusing capacity for carbon monoxide (D_{LCO}) and total severity score (TSS) were used as outcome variables. The association between them and miRs was determined by random forest (RF) for multivariate analyses and generalized additive models (GAMs) for univariate analyses, adjusted for variables such as age, sex, and previous pulmonary disease, to identify miRs that were thought to significantly contribute to pulmonary sequelae [44].

3. miRNA-Based Treatment for ARDS

Overexpressing miRs can be achieved with miR mimics, synthetic double-stranded RNA oligonucleotides, as shown in **Table 1**. Mimics interact with endogenous miR processing machinery and are sorted into RNA-induced silencing complexes (RISCs) to interact with their target transcript. Due to this interaction, minimal chemical modification of the oligonucleotides is required to optimize efficacy. Modifying the passenger strand with 2′-methylated nucleosides ensures that the correct strand is incorporated into RISCs [45]. Ensuring that passenger strands are not also incorporated can reduce the side effects of miR therapy by increasing target specificity [45].

Table 1. Summary of the use of miRNAs in models of lung injury in papers.

	miRNA	Direct Target	Pathway	Target Organ/Cell	Expression When Therapeutic	Carrier	Route	Source
Adverse	miR-155	SOCS1	NF-κB	Macrophages	Downregulated	Exosome	Injected intravenously	[46]
	miR-193b-5p	Occludin	Unknown	BEAS2b, HPMECs and mouse lungs	Downregulated	HiPerfect reagent	Injected intratracheally	[36]
	miR-762	NFIX	miR-762/NFIX	A549 and HEK293T	Downregulated	Lentivirus	Injected intranasally	[47]

	miRNA	Direct Target	Pathway	Target Organ/Cell	Expression When Therapeutic	Carrier	Route	Source
	miR-27a-5p	VAV3	Unknown	Mouse lungs	Downregulated	HiPerfect reagent	Injected intratracheally	[38]
	miR-34b-5p	PGRN	Unknown	Lung homogenates	Downregulated	None	Injected intravenously	[48]
	miR-221	SOCS1	NF-κB	RAW264.7 cells and mouse lungs	Downregulated	None	Injected intravenously	[49]
	miR-126	SPRED1	RAF/ERK	HUVEC and mouse lungs	Upregulated	Exosome	Injected intravenously	[50]
	miR-384-5p	Beclin-1	Possibly Autophagy (Not fully known)	Alveolar macrophages and Mouslungs	Upregulated	Exosome	Injected intravenously and intratracheally	[51]
	miR-371b-5p	PTEN	PI3K/Akt	Human primary ATIICs and mouse lungs	Upregulated	Exosome	Cell experiment	[52]
	miR-125b-5p	Keap1/Nrf2/GPX4	Keap1/Nrf2/GPX4	PMVEC	Upregulated	Lipofectamine	Cell experiment	[53]
Protective	miR-223	PARP-1	NF-κB/AP-1	Mouse lungs	Upregulated	Neutral lipid emulsion (Lipid nanoparticle)	Injected intratracheally	[54]
	miR-23b-3p	FGF2	NF-κB	Mouse lungs and BMSC	Upregulated	Lentivirus	Injected intratracheally	[55]
	miR-127	CDK6	IgG Fcγ Receptor I	RAW264.7	Upregulated	Lentivirus	Cell experiment	[56]
	miR-200c/b	ZEB1/2	p38 MAPK and TGF-β/smad3 (Unknown)	RLE-6TN (rate alveolar cell) and mouse lung	Upregulated	Lentivirus	Injected intratracheally	[57]
	miR-506	p65	NF-κB	Mouse lung	Upregulated	Lentivirus	Injected endotracheally	[58]
	miR-193b-3p	β-catenin	Wnt/β-catenin	AECII and Mouse lung	Upregulated	Adenovirus	Injected intratracheally	[59]
	miR-454	CXCL12	CXCL12/CXCR4	Mouse lung	Upregulated	Adeno-associated virus	Tail vein injection	[60]
	miR-4262	Bcl-2	Unknown	Mouse lung	Upregulated	Adeno-associated virus	Tail vein injection	[61]

Table 2. Summary of miRNAs used in clinical trials.

Drug	miRNA	Drug Type	Carrier	Phase	ClinicalTrials.gov Identifier	Illness	Source
RGLS4326	Anti-miR-17	Locked nucleic acid (LNA) Inhibitor	Unknown	Phase I	NCT04536688	Autosomal dominant polycystic kidney disease	[62]
LNA-i-miR-221	miR-221	Inhibitor	Unknown	Phase I	NCT04811898	Refractory advanced cancer	[63]
MRX34	miR-34a	Mimic	Liposomal nanoparticle	Phase I (Terminated)	NCT01829971	Refractory advanced cancer	[64]

4. The Advantages of miRNA Therapy for Complex Acute Conditions

One of the main advantages of miR-based therapies is that they modulate a naturally occurring regulatory system. Endogenous mechanisms have evolved for the cell to manage exogenous RNA as a host defence from pathogens; by manipulating naturally occurring regulatory systems, miR-based therapies may be less likely to trigger this same host response [65].

Second, the ability of miRs to target multiple transcripts in different pathways may induce a broader response [66][67]. This is important given the complexity and the redundancy of the innate immune response and the heterogeneity of responses to an acute insult [68]. With this intricacy of the inflammatory response in mind, it has become clear that targeting a 'single' mediator is unlikely to effectively change outcomes in the critically ill—likely because of the complex and intricate complementation and compensation circumventing any beneficial effect of single mediator therapy [69]. MiRs target entire networks of genes, which may enable us to stimulate or dampen the activity of a broad network effectively [70][71].

Thirdly, given the severe, disseminated, but transient nature of immune dysregulation during injury, infection, and inflammation, approaches that rely on a "hit-and-run" effect (exposure-response therapy) might be more effective by working quickly to mitigate innate immune dysfunction and reconstitute homeostasis [72].

Alternatively, the effects of an endogenous miR can be mitigated by miR inhibitors [48][49], miR sponges, and molecular inhibitors that impede miR–mRNA interaction. miR inhibitors, chemically modified nucleic acids also called antagomirs or antisense oligonucleotides (ASO), work by binding to miRs via complementary base pairing to form an RNA duplex that will then be degraded by endogenous RNAse H. In addition to pre-clinical studies, anti-miR therapies have been evaluated in Phase I and II clinical trials, as shown in **Table 2**.

Fourthly, it may be possible to tailor therapy to specific miRs with both biomarker and biotarget function (theragnostic). Given delivery vehicles can be manufactured on demand and be used to deliver various miR-based therapeutics simultaneously, combination therapy would be relatively feasible and safe.

Finally, by creating a library of miR-based therapies tailored to address specific treatable traits, designing single or combination miR-based delivery vesicles for individualized therapy may be feasible, making personalized off-the-shelf therapeutics for complex acute care syndromes possible.

5. miRNA Can Be Modified to Optimize Delivery

As oligonucleotides, miR-based therapies must overcome degradation and avoid immune stimulation. This can be done by modifying the 2′-OH of the ribose sugar backbone, such as a 2′-*O*-methyl group, which does not affect miRNA mimic function [73][74][75].

Additionally, chemically modifying miRs allows for direct manipulation of pharmacokinetic and pharmacodynamic properties, which can be used to optimize dosing and therapeutic ranges. The dosing of miR-based therapies significantly affects target interaction and off-target effects [76]. The effects of an miRNA-based therapy may be investigated through miR knockout transgenic mice lines and delivering the miR inhibitor as a control. Subsequently, RNA can be profiled using transcriptomics. While these strategies address the effects that one miR may have on a variety of target transcripts, it is also true that one transcript can be acted upon by more than one miR [77]. Therefore, it can be challenging to predict the extent to which a given dose of miR-based therapy will induce target gene silencing. It is also essential to recognize that miR-based treatments rely on uptake by endogenous RISCs, which can lead to saturation and subsequent interference with other endogenous miR pathways [78][79].

6. Vectors Can Be Used for miRNA Delivery

As discussed, miRs are prone to extracellular degradation, off-target complementary binding, and immune stimulation. For this reason, the vector used to deliver miR therapeutics can significantly impact efficacy, dosing, and toxicity. Vectors used for miR delivery include lipid nanoparticles, exosomes, and viral vectors.

7. Lipid Nanoparticles

Lipid nanoparticles (LNPs) are spherical membranes composed of ionizable lipids, which are positively charged at low pH and neutral at physiological pH environments. LNP lipid composition promotes plasma membrane interaction and facilitates systemic cellular uptake via receptor-mediated endocytosis. The use of lipid nanoparticles in mRNA SARS-CoV-2 vaccines demonstrates the potential of LNPs as vectors for nucleic acid delivery. Moreover, extensive research and now widespread use of LNP technology can significantly reduce the burden of seeking pre-clinical approval, as demonstrated by the expedited roll-out of the Pfizer-BioNTech (BNT162b2) and Moderna (mRNA-1273) SARS-CoV-2 vaccines.

All FDA-approved LNP particles contain an ionizable cationic lipid, helper lipids, cholesterol, and polyethylene glycol (PEG)-lipid conjugates [80][81][82], each contributing to LNP size, stability, cellular uptake, and other important factors that impact vector efficacy [83]. A commonly used lipid vector is the liposome. Liposomes can be cationic; however, the positive charge can be toxic in high doses, promote inflammation, and interact with endogenous negatively charged proteins.

PEG-lipid components improve the half-life of circulating LNPs [84], but a high concentration of PEG-lipid in the LNP can impede endocytosis [85]. For this reason, the concentration of PEG-lipid in the LNP is minimized as much as possible; Semple et al. aimed to optimize cationic lipids for siRNA delivery and noted vital findings such as optimal pKa constants for LNPs, and ideal PEG-to-ionizable lipid component ratios [86]. PEG-lipids that can diffuse out of the LNP have also been explored, demonstrating a lengthened circulation time [87][88][89]. In contrast, permanently PEGylated LNPs are more immunogenic, resulting in rapid clearance, an antibody-driven reaction, and reduced potency upon subsequent exposure [90][91].

8. Extracellular Vesicles

Extracellular vesicles (EVs) are endogenous cell-to-cell communicators that have also been explored as carriers of oligonucleotide-based therapies. Exosomes are a subset of EVs, and can emerge from nearly all cell types. Exosomes are formed via invagination of endosomal membranes, creating multivesicular bodies (MVBs) [92]. They are then taken up through fusion of the MVBs and target cell plasma membranes. Exosomes are composed of lipids and surface proteins from their origin cells. This cell-specific lipid and protein composition can affect targeting and distribution. Transporter proteins such as CD13, and fusion proteins including flotillin and annexin are key proteins that can differ depending on the cell source and dictate target cell delivery [93].

Endogenous exosomes carry a variety of cargo, including miR [94]. One such example includes miR-317b-5p, carried in exosomes derived from alveolar progenitor type II cells (ATIIC) in the lungs, which was found to promote re-epithelialization of injured alveoli and modify ATIIC proliferation in an in vitro model of alveolar injury [52].

9. Viral Vectors

Viruses are another group of delivery vectors for miR-based therapeutics [95]. To carry genetic material, the critical viral genes are replaced with the desired transgene, including miRs [96]. Types of viral vectors used in laboratory or clinical settings to carry miRs include adenoviruses (Ad), adeno-associated viruses (AAV), herpes viruses, poxviruses, retroviruses, and lentiviruses [52][57][95]. One study used a retrovirus vector to deliver NF-κB p65 siRNA in a sepsis-induced ALI mouse model [97]. In the case of retrovirus-based vectors, the main limitation is that replicating their cargo and delivering the gene therapy requires cell proliferation, which is not always present with lung cells [98]. Alternatively, lentiviral vectors can infect a range of dividing and non-dividing cells, integrating into the host genome stably without affecting their normal function [99]. Lentiviral vectors contain plasmids that fall into two parts: packaging system and transfer vector [99]. The packaging system is required for viral particle formation and infectivity, while the transfer vector is required for mobilizing the viral genome [99]. The transfer vector contains the transgene and cis-acting sequences for RNA production and packaging [99]. Lentiviral vectors have been used to deliver miR-127 to reduce CD64 expression and lung inflammation [56]. Lentivirus-carrying miR-23b-3p or miR-762 inhibitors have been used to promote lung repair and reduce lung injury characterized by alveolar epithelial cell destruction, increased blood-air barrier permeability, and non-cardiogenic pulmonary edema [47][55]. Despite some degree of success in preclinical models, safety concerns—the most pressing of which is the activation of protooncogenes—remain [100].

Ad and AAV-derived vectors are the most studied and commonly used viral vectors [101]. AAV vectors have a protein shell that protects a single-stranded genome with three genes, producing nine gene products essential for genome replication, packaging, and cell binding [102]. AAV depends on co-infection with other viruses for replication, and hundreds of unique strains across different species are known [102]. Recombinant AAV (rAAV), which lacks viral DNA, can be engineered to deliver an oligonucleotide cargo to the nucleus of a cell [102]. The advantages of rAAVs include low pathogenicity, low immunogenicity, low risk for insertional effects, and the ability to infect both dividing and non-dividing cells in a broad spectrum of tissues [99][101].

10. Delivery to Lungs

Most clinically tested miR therapeutic candidates are delivered through intramuscular or intravenous injection [67]. While systemic administration has been shown to provide treatment to the lungs, direct pulmonary routes of administration may mitigate off-target effects and increase bioavailability at the target site.

While intratracheal and intranasal delivery methods still have clinical relevance, intranasal administration is hindered by filtration in the human nasal cavity, and the invasive nature of intratracheal administration limits generalizability. Though less common, animal models using inhalation have demonstrated the efficacy of oligonucleotide therapeutics [103][104].

Inhalation delivery of RNAi therapeutics requires careful consideration of vector composition. While naked siRNA and mRNA have been successfully delivered to the lungs, it is essential to recognize that this is only effective for specific cell types that reside in the lung [105][106][107].

11. Lipid-Based Vectors for miR Delivery to Lungs

In direct pulmonary administration, a limitation to lipid-based vectors is structural stability due to fusion with lung surfactant, resulting in premature RNA release [108]. Specific composition profiles of LNPs have been shown to improve stability, as is demonstrated by MRT5005 clinical studies transfecting mRNA via nebulization [109].

Advancements in direct lung delivery of mRNA via nebulization, in which the medication is aerosolized and inhaled, have shown promise [110]. One study compared different LNP compositions and molar ratios to define optimal LNP makeup for low-dose delivery of mRNA to the lungs via nebulization in mice, which differed from optimization for systemic delivery [111]. This method was tested in an H1N1 mouse model, in which mRNA for a neutralizing antibody was loaded on the LNP vector [111]. PEG molarity, density, and structure significantly impacted delivery performance, as well as the inclusion of cationic lipids [111].

Nebulized formulations of LNPs can have advantages for treatments for pulmonary targeting. Compared to naked oligonucleotides, LNPs greatly decrease RNase-mediated degradation and ensure a significant proportion of the therapeutic dose or active therapeutic ingredient is delivered to the lungs, thus exposing affected tissues to greater therapeutic concentrations of the product [112].

Furthermore, the deposition of inhaled substances in the respiratory tract depends on various factors, with the aerodynamic diameter (AD) of the nebulized formulations playing a key role in where it will end up along the respiratory conducting and non-conducting zones [113][114]. LNPs with an AD between 1 and 5 μm are more likely to settle in the lower airways, reaching even the farthest bronchioles and alveoli due to gravitational sedimentation. This is conducive for deeper penetration into lung tissue [114][115].

12. Delivering Exosomes to the Lungs

Careful selection of exosome subtypes and genetic modification of exosome membrane proteins can facilitate targeted biodistribution [116]. Exosomes used in pre-clinical studies have successfully delivered miR to the lungs in lung injury disease models. For example, intravenous delivery of miR-155 results in macrophage activation and subsequent lung injury in mice [46]. Additionally, intravenously administered bone marrow mesenchymal stem cell (BMSC)-derived exosomes containing miR-384-5p improved pulmonary vascular permeability in a rat model of LPS-induced ALI [51].

Alternatively, direct delivery of extracellular vesicles can also target therapy to the lungs. Bandeira et al. demonstrated that intratracheally delivered extracellular vesicles obtained from MSCs mitigated fibrosis and reduced inflammation in the lungs of a silicosis mouse model [117].

13. A Few Words about Silencing-(si)RNA-Based Therapies

RNAi involves double-stranded RNA that silences mRNA via complementary binding, resulting in post-transcriptional gene regulation. RNAi includes miR and siRNA, which are both generated by double-stranded RNA processed by Dicer, and incorporated into RISC with Ago2 interaction. miR and siRNA also share many physical properties as they are both oligonucleotides. These connections between miR and siRNA implicate similar advantages and barriers in therapeutic delivery, including extracellular degradation of nucleic acids and strategies of lung localization.

One key differentiating factor between siRNA and miR is that siRNAs are specific to a single mRNA transcript while miRs can act on multiple transcripts in different cellular pathways. This difference is explained by the full complementarity of siRNA to the target mRNA, contrasting the complementarity of miR to shared target seed sequences.

Furthermore, miRs can be used as disease biomarkers as endogenous host entities. A shared barrier of RNAi includes the reliance on endogenous RISC uptake, which can result in the saturation of this machinery and subsequently impede endogenous miRNA pathways. The off-target effects in the perturbations of endogenous miR via RISC saturation can be more unpredictable, making dosing in RNAi-based therapies an essential factor in risk assessment.

Interestingly, siRNA-based treatments may have ‘miR-like effects’ when an siRNA binds the 3′UTR of an off-target mRNA [118][119]. This can often be avoided with more careful siRNA design, such as preventing complementarity to miRNA seed regions already identified in miRNA databases.

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