The absence of collagen and elastin production by lung mesenchymal stem cells demonstrated by pentachrome stain.
Role of microRNAs in lung development and pulmonary diseases

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ABSTRACT

MicroRNAs (miRNAs) are a class of small noncoding RNA which exert post-transcriptional gene regulation activity by targeting messenger RNAs. miRNAs have been found to be involved in various fundamental biological processes and deregulation of miRNAs is known to result in pathological conditions. In this review, we provide an overview of recent discoveries on the role played by this class of molecules in lung development and in pulmonary diseases, such as asthma, cystic fibrosis, chronic obstructive pulmonary disease, and pulmonary artery hypertension. Considering the relevant role of these miRNAs under physiological and pathological conditions, they represent new clinical targets as well as diagnostic and prognostic tools. Therefore, this review pays special attention to recent advances and possible future directions for the use of miRNAs for clinical applications.

Key Words: asthma, COPD, cystic fibrosis, microRNA, pulmonary arterial hypertension, pulmonary development, pulmonary disease

Significant development has been made towards understanding the contribution of microRNAs (miRNAs) in development of lung and pathogenesis of pulmonary diseases.[1,2] This review will provide an overview on the role of miRNAs in lung disease reassuming recent findings that extend our perspective on the importance of these molecules in guiding the cellular response upon different stress conditions. Interestingly, it appears that this class of non-coding regulatory RNA play a key role in several aspects of inflammation which is a determinant feature in many lung diseases such as cystic fibrosis, emphysema, asthma and Chronic Obstructive Pulmonary Disease (COPD).[3] It is believed that miRNAs are involved in various aspects of innate and acquired immunity, and therefore, indirectly involved in these inflammatory lung diseases.[4,5] In this review, we will discuss the involvement of miRNAs in lung development as well as the pathogenesis of various pulmonary diseases (Table 1).

MICORRNA BIOGENESIS AND FUNCTION

miRNAs are small noncoding RNAs that function by regulating target gene expression post-transcriptionally. The first miRNAs lin-4 and let-7[7,8] were discovered in 1993 and 2000 respectively in Caenorhabditis elegans (C. elegans) as genes involved in developmental timing by mediating sequence specific post-transcriptional regulation of gene expression. Initially this new class of RNA was thought to be present only in C. elegans, however, it was quickly revealed that it exists in many different animal species, including mammals.[9,10] Spatially and chronologically restricted expression patterns, and evolutionary conservation of miRNAs suggested that they have a broad regulatory function in plants and animals. It has been shown that the whole miRNA system regulates greater than 60% of protein-coding genes in a human cell.[11] Close to 2000 human miRNAs have been described to date (Sanger miRBase version 19, http://www.mirbase.org/blog/2012/08/mirbase-19-released/), which play critical roles in development and physiological and pathological processes.

miRNAs are mainly encoded in either inter-genic regions or within protein coding genes, usually introns. The molecular origins of miRNAs and mRNA targets are not well
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miRNAs have rapidly emerged not only as critically

evaluated, however, it is suggested that miRNAs originate
from transposable elements.[12,13] Indeed, several miRNA loci
have been described at a repetitive element transposition and
subsequent transcription across two tandems, inverted
repeat elements.[14] Alternatively, miRNAs in plants seem to
be originated from inverted duplication event of the target
gene that can form a self-complementary hairpin with the
potential to spawn in new miRNA genes if it can confer an
advantage of positive selection.[15,16]

The biogenesis of miRNAs begins with the transcription of
the primary transcript (pri-miRNA) of about few hundreds
to few thousand nucleotides in length (Fig. 1). RNA
polymerase II is involved in the production of this capped
and polyadenylated stem-loop-structured pri-miRNA,
even though it has been found that some pri-miRNAs, in
particular those that lie within the Alu repeats, can be
produced by RNA polymerase III.[14] Subsequently the
pri-miRNA undergoes processing by RNA endonucleases
(RNase) in two sequential cleavage steps. The first
cleavage takes place in the nucleus by the type III RNase
Drosha that liberates a shorter oligonucleotide of about
~70-90 nucleotide (nt) in length called precursor-miRNA
(pre-miRNA). Drosha cleaves pri-miRNAs together with
a cofactor known as DiGeorge syndrome critical region
gene 8 (DGCR8) which represents an important hub for
many regulatory proteins involved in the regulation of the
miRNA maturation process.[18,19](Fig. 1). The pre-miRNA is
then exported to the cytoplasm by the Exportin 5 protein
where it is further processed by the RNase III enzyme Dicer.
Dicer generates a ~22 nt double-strand ribonucleotides
with 5’ phosphate and two nucleotides 3’ overhangs
(Fig. 1). In the subsequent step, the two strands are
separated and one of them is recruited by the Argonaute
(Ago) proteins[21] to form a multi-protein complex known
as the RNA-induced silencing complex (RISC)[22](Fig. 1).
This loaded RISC machinery is able to guide the miRNA to
target partially complementary sequences, usually present
in the 3’-untranslated region (UTR), on mRNA transcripts.
Generally, just one strand of miRNA is accumulated inside
the cell and loaded in the RISC complex by association
with Ago1 and Ago2 which are the two most highly
expressed Ago proteins present in mammalian cells.[23] The
other strand, often referred as “miRNA*”, was originally
considered as a byproduct of the miRNA biogenesis,
historical recent findings demonstrated that it retains
biological activities.[24-28] The 6-8 nucleotide sequence at the
5’-end of miRNA which contributes to base pairing with the
target miRNAs is referred as the “seed sequence”. In recent
years contrasting results have been published regarding the
mechanism of miRNA binding to Ago proteins. Ago2 is the
is the only isoform with RNase activity and mainly acts to
promote mRNA degradation while Ago1 seems optimized
for cleavage-independent repression.[29] Furthermore,
Ago2 appears to have selectivity for siRNA-like duplexes
with more extensive base pairing.[29,30] It is noted that miR*
strands represent an abundant fraction of RNA species
bound to Ago2.[31] Therefore, miR:miR* duplex could be
bifunctional with one strand associated with Ago1 which
functions as a cleavage-independent repression of the
mRNA, and the miR* associated with Ago2 which triggers
a cleavage-dependent repression of the miRNA.[32]

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Table 1: miRNAs in lung disease

| COPD    | miR-223, miR-1274[18] | Up     |
|         | miR-923, miR-937, miR-125b-1 | Down   |
|         | miR-101 and miR-144[51] | Up     |
|         | miR-146a[45] | Up     |
|         | miR-1452[83] | Down   |
|         | suggested paper on miRNA screening related to COPD[16,67] and CS[63,91] |         |
| Asthma  | miR-126[90] | Up     |
|         | miR-145a[79] | Up     |
|         | miR-146a; miR-146b; miR-181[192] | Down   |
|         | let-7[94] | Up     |
|         | miR-21[76] | Down   |
|         | miR-221; miR-222[83] | Up     |
|         | miR-106a[81] | Up     |
|         | miR-20b[84] | Up     |
|         | miR-155[193] | Down   |
|         | miR-133a[104] | Down   |
| Cystic fibrosis | miR-155[107,109,195] | Up     |
|         | miR-101; miR-494[103] | Up     |
|         | miR-126[100] | Down   |
|         | miR-138[106] | Down   |
| Idiopathic Pulmonary Fibrosis | miR-29[124] | Up     |
|         | miR-2[177] | Down   |
|         | miR-154[122] | Up     |
|         | let-7d[120] | Down   |
|         | miR-17[122] | Down   |
|         | miR-155; miR-125b[122] | Up     |
|         | suggested paper on miRNA screening in bleomycin-induce lung fibrosis[172] |         |
| PAH     | miR-17-92[123,146,148] | Up     |
|         | miR-206[187] | Down   |
|         | miR-143-145[157] | Up     |
|         | miR-21[155,154,154] | Up     |
|         | miR-27a[144] | Up     |
|         | miR-204[141,149] | Down   |
|         | miR-210[147,148] | Down   |
|         | miR-424[149] | Down   |
|         | miR-503[148] | Down   |
|         | miR-150[135] | Down   |
| Acute lung Injury | miR-127[173] | Down   |
|         | miR-21; miR-155; miR-146[170] | Down   |
|         | miR-32*; miR-466-5p; miR-466-3p[171] | Down   |

List of miRNAs that are differentially expressed in lung pathologies, as reported in the literature. Up and Down indicate if the miRNA is elevated or reduced in diseased tissues, respectively; COPD: chronic obstructive pulmonary disease; PAH: pulmonary artery hypertension.
contributing to various pulmonary diseases but also to normal development of the lung and maintenance of its homeostasis. Lung development can be arbitrarily divided into 6 stages that are found in all mammalian species: embryonic, glandular, canalicular, saccular, alveolar and vascular maturation. After the initial differentiation of lung epithelial cells from the endoderm, the epithelial tube is formed with little to no lumen in the glandular phase. In the canalicular phase, bronchioles are produced and undergo thinning of the interstitium and a flattening of the epithelium in order to form alveolar ducts and air sacs during the saccular phase. At the final stage, alveoli are formed after birth.

Fetal lung development is a complex process temporally and spatially orchestrated by a complex set of many genes. The signalling molecules involved in lung development include fibroblast growth factors (FGFs), transforming growth factor-β (TGF-β), Wnts, and hedgehogs. The evidence that miRNAs play a role in lung development derive from mice with conditional knockout of Dicer in lung epithelial cells that exhibit a failure of epithelial branching, underscoring the essential regulatory role of miRNAs in lung epithelial morphogenesis. At embryonic stage 11.5 (E11.5) in the lung epithelial branching region, Ago1 and Ago2 are enriched in distal epithelium and mesenchyme respectively, further underscoring the important role of miRNAs in the dynamic changes occurring during lung remodeling and maturation. Evaluation of miRNA expression patterns during the different phases of lung development revealed 27 miRNAs which are differentially expressed. For instance miR-29a plays a role in limiting proliferation in the late stage of lung development. It is also highly expressed in the adult lung and from over-expression in lung cancer cells appears to inhibit tumorigenicity. The expression of the miR-17~92 cluster, which includes 6 miRNA genes (miR-17, -18a, -19a, -20a, -19b-1, and -92-1), is decreased as development progresses. An gene ontology (GO) analysis of the predicted targets of this cluster show that these miRNAs control genes which are critical for proper developmental progress, such as transcriptional factors and regulators of nucleic acid metabolism. The overexpression of this cluster is observed in lung cancer cells. Studies on the role of miR-17~92 locus in lung development have found that miRNAs in this locus are highly expressed in undifferentiated lung progenitor epithelial cells and, by regulating the cell cycle regulator gene Retinoblastoma-like 2 (Rbl2), it promotes their proliferation. In contrast, miR-127 and miR-351 are expressed transiently during...
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In this section, we will discuss the link between deregulation of miRNAs and the pathogenesis of different pulmonary diseases (Table 1) as well as the potential therapeutic approaches based on restoration of the physiological miRNA signature.

Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) presents as the reduction of airflow due to abnormalities in the small airways compartment and associated infiltration of inflammatory cells, thickening of the alveolar wall and ultimately damage of the lung parenchymal and epithelium. It encompasses both emphysema and chronic asthmatic bronchitis. Emphysema results from progressive alveolar septal destruction with concomitant reduction of the surface area available for gas exchange, while bronchitis constricts the airways clogging them with production is induced by pro-inflammatory cytokines such as IL-1β and TNF-α and depends on the expression of COX-2 together with the expression of COX-2 takes place both in COPD and control fibroblast, but to a lesser extend in COPD fibroblast. In this context miR-146a is thought to be a feedback control mechanism that limits the intensity and duration of inflammation by reducing the level of COX2 and hence the production of PGE2.

A regulatory role of miRNAs has also been observed in the fibroblasts of tissue specimens derived from smokers affected by COPD who underwent surgery. This experiments focused on the role of prostaglandin (PG) E2, since it is an important mediator of tissue inflammation that increases in expression in the lungs of patients with COPD. Its production is induced by pro-inflammatory cytokines such as IL-1β and TNF-α and depends on the expression of COX-2 together with the expression of COX-2 takes place both in COPD and control fibroblast, but to a lesser extend in COPD fibroblast. In this context miR-146a is thought to be a feedback control mechanism that limits the intensity and duration of inflammation by reducing the level of COX2 and hence the production of PGE2.

Recently the search for biomarkers for early detection of COPD has drawn attention on the role of circulating miRNAs. A study found five deregulated circulating miRNAs in serum of COPD patients, four of them downregulated (miR-20, miR-28-3p, miR-34c-5p and miR-100) and one upregulated (miR-7) in comparison with healthy individuals. Finally, similar attempts to apply miRNA expression signature as
a diagnostic tool, suggest the possibility of distinguishing COPD patients from lung cancer patients by measuring their miRNA expression pattern.\cite{68,69}

**Asthma**

Asthma is a lung condition that is increasingly prevalent in the western world, which is triggered by a sustained inflammatory response in the airway that is caused by a combination of air pollutants and genetic predisposition. Asthma is characterized by reversible airway obstruction, peribronchial inflammation and airway hyperresponsiveness (AHR). A relevant feature of asthma is the occurrence of acute exacerbation events that involve enhanced contraction of the airway smooth muscle layer in response to inhaled stimuli and are the consequence of AHR. In the late stages of the disease, airway remodelling occurs which consists of epithelial desquamation, goblet cells hyperplasia, smooth muscle cell hypertrophy and hyperplasia, an increase in matrix protein deposition, basement membrane thickening and growth, and proliferation of new blood vessels.\cite{70} The inflammatory response plays a pivotal role in the progression and pathology of asthma featuring a production of IgE and involving a consistent recruitment of leukocytes, in particular eosinophils, together with type 2 T-helper (Th2) cells and mast cells.\cite{71} TGF-β signalling has been found to play a key role in the remodelling that occurs during the progression of asthma.\cite{72} Indeed TGF-β, in addition to its pro-fibrotic action, characterized by increased synthesis of collagen\cite{73} and fibronectin,\cite{74} also promotes airway smooth muscle cell proliferation and epithelial apoptosis.\cite{75}

Several aspects of asthma have been linked to the deregulation of miRNAs (Table 1). Of particular interest is the involvement of miRNAs in the regulation of the immune response and inflammation by targeting several immune receptors and cytokines. In this context, miR-21 has been described to target IL-12 which is a major cytokine involved in Th1 cell polarization.\cite{76} MiR-21 ablation in mice challenged with ovalbumin has been described to mitigate the progression of the pathology by alleviating the allergic immune response and skewing the balance between Th1 and Th2 responses toward Th1.\cite{77} In a opposite way, a cell polarization toward a Th2 response has been observed to be concomitant with the down modulation of let-7 family in inflamed airways.\cite{78} Exogenous administration of let-7 in asthmatic mice was shown to reduce the level of IL-13, an important mediator of the Th2 response during allergic events.\cite{79} An additional miRNA involved in the Th2 response and eosinophil recruitment is miR-145.\cite{79} Mice challenged with House Dust Mite (HDM) allergen and then treated with miR-145 inhibitor displayed a reduction of pro-inflammation cytokines, IL-13 and IL-5, and alleviation of asthma symptoms. The anti-inflammatory effects of miR-145 inhibitor were comparable to those of steroid treatment.\cite{79} T lymphocyte polarization is also regulated by miR-126, which is a potent and specific inducer of the Th2 allergic response and TLR4 signalling present in asthma.\cite{80} Exposing the airways to a miR-126 inhibitor abolished HDM induced airway hyper responsiveness and attenuated mucus hyper-secretion and accumulation of eosinophils.\cite{80} Beneficial effects have also been obtained by knocking down miR-106a in the ovalbumin sensitized murine model of asthma; in this experiment knock-down of miR-106a increased the level of the anti-inflammatory cytokine IL-10 and reduced the Th2 response alleviating AHR.\cite{81} The anti-inflammatory role of IL-10 is also dependent on its role in counteracting the LPS mediated induction of miR-155, which increases PIP3 levels and activates the downstream signalling of NF-KB and MAPK.\cite{82} Studies have also been performed that address the role of mast cells in asthma. The miR-221, -222 family was demonstrated to be transcriptionally induced upon mast cell activation. In particular miR-221 favors mast cell activation upon IgE-antigen complex stimulation by promoting cell adherence, cytokine production and degranulation.\cite{83}

A comprehensive study of miRNAs differentially expressed in lung tissue of mice challenged with ovoalbumin – a model recapitulating the main phases of asthma progression – found 58, 66 and 75 miRNAs significantly modulated by short, intermediate and long-term challenge respectively.\cite{84} Bioinformatics analysis of the data coming from this study found out miRNA-regulators of several pathways ranging from cell cycle, TGF-β, protein synthesis and inflammation. Interestingly, cell proliferation is more pronounced in the early stages of asthma than in the late ones. MiR-574-5p is involved in cell proliferation and inversely correlated with NuSAP, a microtubule-associate protein involved in mitotic spindle organization.\cite{85} MiR-29b and miR-29c, which are correlated with matrix deposition and target MMPs, are downregulated in the late stages. Notably miR-146a, which is regulated by Toll Like Receptors (TLRs) was the only miRNA found to be regulated in all three phases.\cite{84} Forced expression of miR-146a decreases the production of various pro-inflammatory cytokines and chemokines.\cite{86,87} Moreover, T cell receptor driven NF-kB activation of miR-146a is important in down-regulating NF-kB activity in a negative feedback loop, at least partly through repressing the NF-kB signalling transducers TRAF6 and IRAK1, thus modulating the T-cell immune response.\cite{88}

It is worth mentioning that increasing evidence suggests that angiogenesis plays an important role in the progression of asthma.\cite{89,90} Vascular endothelial growth factor (VEGF) levels are increased in tissues obtained from asthmatic subjects and it has been demonstrated that alveolar epithelial cells,\cite{91} fibroblast\cite{92} and leukocytes\cite{93} produce VEGF during the inflammatory process. Alveolar
macrophages (AM) are an important source of VEGF during lung injury. Increased miR-20b expression in AM from asthmatic mice decreases VEGF protein levels.\textsuperscript{[94]} Analysis of the contribution of the vasculature to the establishment of the aberrant inflammation response may lead to new insights in the treatment of asthma. For instance, overexpression of miR-181b in endothelial cell inhibits NF-kB nuclear localization and signalling which down-modulates leukocyte influx hence decreasing lung injury.\textsuperscript{[95]} This discovery suggests that miR-181b may be a new target for anti-inflammatory therapy.

**Cystic fibrosis**

Cystic Fibrosis (CF) is a monogenic lung disease\textsuperscript{[96]} characterized by mucus airway obstruction, neutrophil-dominated airway inflammation and bacterial infection that leads to progressive pulmonary damage and emphysema.\textsuperscript{[97]} The gene involved in the insurgence of CF is the CFTR encoding for an ATP-dependent anion channel-protein present in the apical part of the epithelium. CFTR coordinates the secretion and absorption of NaCl, fluid and airway surface liquid homeostasis.\textsuperscript{[98]} The most common CFTR mutation causes erroneous protein folding and degradation, which ultimately leads to an imbalance between Cl secretion/Na\textsuperscript{+} absorption and airway surface liquid depletion. As a consequence of the epithelial layer dehydration, mucociliary clearance is impaired which affects innate immune defences favouring infection by opportunistic pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*.\textsuperscript{[99]} Neutrophil-dominated airway inflammation has been implicated as a driving force in the airway remodelling and bronchiectasis. These processes, characterized by bronchial wall thickening and tissues fibrosis, are mediated by the production of reactive oxygen species and metallo-proteases.

The first work describing miRNAs involvement in CF was published by Oglesby et. al.\textsuperscript{[6,100]} (Table 1). In this work, miR-126 was shown to target TOM1 protein, a known regulator of endosomal trafficking of ubiquitinated proteins,\textsuperscript{[101]} which is also a negative regulator of IL-1β, TNF-α and LPS signalling pathway.\textsuperscript{[102]} miR-126, which is highly expressed in the lung, is down modulated in the epithelium of CF patients; this leads to increased expression of TOM1 and decreased NF-kB-induced cytokines, such as IL-8.\textsuperscript{[100]} This mechanism may be an attempt of the lung epithelium to compensate for the high pro-inflammatory burden of CF.\textsuperscript{[6]} miRNA Responsive elements (MRE) for miR-101 and miR-494 have been identified in the 3’UTR of the CFTR transcripts these miRNAs have been shown to inhibit CFTR translation.\textsuperscript{[103]} Notably, these two miRNAs are elevated in CF patients and their alteration has been linked to other changes in pro-inflammatory cytokines, cells cycle progression and carcinogenesis.\textsuperscript{[104-106]} CFTR is also indirectly regulated by miR-138, which targets the transcriptional regulatory protein SIN3A. MiR-138 relieves the transcriptional repression of SIN3A and allows the expression of wild type and mutant form of CFTR restoring Cl transport in CF epithelia.\textsuperscript{[91]}

In vitro comparison of differentially expressed miRNAs between CF epithelial cells and control epithelial cells found miR-155 to be highly elevated in diseased tissues\textsuperscript{[107]} (Table 1). The same trend is mirrored in CF lung epithelial cells and circulating CF neutrophils.\textsuperscript{[107]} MiR-155 specifically inhibits Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1 (SHIP1) expression, thereby promoting activation of the PI3K/Akt signalling pathway and promoting expression of the pro-inflammatory IL-8 cytokine\textsuperscript{[107]} – the well-characterized chemotactic agent for neutrophils.

Advances in the care of patient with CF have lead to a dramatic increase in patient survival and a concomitant observation of additional complications, such as tumorigenesis in other organs ascribable to mutation and loss of expression of CFTR. Interestingly, the tumor suppression activity of CFTR is at least in part mediated by the up modulation of miR-193b\textsuperscript{[108]} that in turn targets urokinase-type plasminogen activator (uPA) known to be involved in various malignant traits of cancer.\textsuperscript{[109]}

**Idiopathic pulmonary fibrosis**

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive fibrotic interstitial pneumonia of unknown causes characterized by a rapid progression over a few years.\textsuperscript{[110]} However, several IPF risk factors are known; these include viral infection, occupational activities associated with a high probability of dust or vapour inhalation,\textsuperscript{[111]} cigarette smoke\textsuperscript{[112]} and pollutants.\textsuperscript{[111]} IPF appears to be initially triggered by persistent and repetitive microinjuries to the alveolar epithelium.\textsuperscript{[113]} Continuous damage of the epithelial cells leads to the destruction of the basal membrane and hence the activation of coagulation and metalloproteinases. Later steps of IPF include the massive deposition of extracellular matrix by activated myofibroblasts derived from different sources, such as fibroblast, pericytes, alveolar epithelial cells and mesothelial cells. IPF is characterized by aberrant activation of developmental signalling pathways, such WNT/β-catenin,\textsuperscript{[114]} TGF-β\textsuperscript{[115]} and Sonic hedgehog.\textsuperscript{[116]} TGF-β is considered the driving force of epithelial-to-mesenchymal transition (EMT).\textsuperscript{[117]} Cells expressing both epithelial and mesenchymal markers have been found in mice treated with TGF-β\textsuperscript{[118]} inhalation. In vitro stimulation of alveolar epithelial cells with TGF-β can induce EMT.\textsuperscript{[119]} The first miRNA demonstrated to be involved in the pathogenesis of IPF was let-7d.\textsuperscript{[120]} Let-7d is downregulated in samples from IPF patients, which was sufficient to cause EMT in lung epithelial cells\textsuperscript{[120]} (Table 1). Decreased let-7d is mediated by TGF-β via a SMAD-dependent mechanism.\textsuperscript{[120]} Inhibition of let-7d
triggers a global decrease in lung epithelial markers and a concomitant increase in alveolar septal thickening.\textsuperscript{[120]}

Levels of miRNAs have been evaluated in different RNA samples from biopsies of IPF patients with varying speeds of disease progression (Table 1).\textsuperscript{[121]} Generally, both slowly and rapidly progressive IPF biopsies revealed a downmodulation of several miRNAs compared to normal tissue and specific miRNAs signature can provide predictive clues about the progression rate of the disease.\textsuperscript{[122]} Interestingly, many upregulated mRNA found in IPF are implicated in EMT and correlate with the downmodulation of the respective targeting miRNA.\textsuperscript{[122]} Furthermore, a decrease in key RISC components such as AGO1 and AGO2 has been observed. Another study found that 43 miRNAs are significantly upregulated in lung tissues from IPF patients and that the majority of them belong to the miR-155 family present as a cluster on chromosome 14.\textsuperscript{[123]} Upregulation of miR-155 was triggered in vitro upon stimulation with TGF-β. Finally, biochemical studies confirmed a mitogenic effect of TGF-β on fibroblast mediated by miR-155 and WNT/β-catenin.\textsuperscript{[123]} Two additionally studies connecting TGF-β signalling to miRNA regulation in IPF have been performed on miR-21 and miR-29. Mice with bleomycin-induced fibrosis present increased level of miR-21 in the lungs that is primarily localized in myofibroblasts.\textsuperscript{[77]} MiR-21 upregulation is induced by TGF-β and promotes the activation of pulmonary fibroblast by targeting SMAD7, thus functioning in an amplifying circuit to promote lung fibrosis.\textsuperscript{[77]} Reciprocally, miR-29 is suppressed by TGF-β in human fetal lung fibroblast. Knockdown of miR-29 derepressed extracellular matrix-associated genes and several fibrosis-related genes, including collagens, laminins and integrins.\textsuperscript{[124]} Restoration of miRNA expression by targeting miRNAs up regulated during IPF may represent a valuable novel therapeutics treatment for this pathology.

Pulmonary artery hypertension

Pulmonary Artery Hypertension (PAH) is a disease characterized by hyperproliferation and neointima formation leading to vasoconstriction of the pulmonary artery and ultimately right ventricular heart failure and death. Both pulmonary artery smooth muscle cells (PASMCs) and pulmonary artery endothelial cells (PAECs) are involved in this disease and extensive studies have been performed on these cells looking for the aberrant molecular mechanisms responsible for the establishment of PAH. Regarding heritable PAH, which is the most frequent form of the disease, in almost 70% of cases the genetic alteration is represented by an autosomal dominant mutation in the bone morphogenetic protein receptor 2 (BMPR2) in PASMC.\textsuperscript{[125]} More rarely, mutations of Endoglin and activin receptor-like kinase 1 (ALK1) have been identified.\textsuperscript{[126]} However, since mutation of BMPR2 occur in just slightly more than half of the cases of heritable PAH it has been suggested that this mutation may cause susceptibility to other factors. Indeed, genetic and environmental stimuli such as dysregulated inflammation,\textsuperscript{[127]} hypoxia\textsuperscript{[128]} and aberrant Notch3\textsuperscript{[129]} and TGF-β\textsuperscript{[130]} signalling can promote the insurgence of PAH.

A first clue that suggested that miRNAs play a role in the pathogenesis of PAH derived from the observation that BMPR2 protein, but not its messenger, is reduced in PAH animal models. From this initial observation it has been found that IL-6 induces the expression of the miR-17-92 cluster through STAT3 activation and that several miRNAs from this cluster target BMPR2\textsuperscript{[131]} (Table 1). Caruso et al. reported a screening of differentially expressed miRNAs in the monocrotaline and hypoxia induced rat models of PAH.\textsuperscript{[132]} In this study the authors found miRNAs similarly deregulated between the two PAH models, in addition they also discovered specific miRNA signature in each group. The discovery of differentially regulated miRNAs between hypoxia and monocrotaline treated rats is in agreement with the differential pathobiology found in the two models. Indeed, monocrotaline challenge mainly targets the pulmonary vascular endothelium triggering strong inflammation while hypoxia stimulates changes also in medial and adventitial with less inflammation.\textsuperscript{[133,134]} Moreover, researchers have identified specific changes in miRNAs signature during PAH progression.\textsuperscript{[135]} An additional screening on deregulated miRNAs in circulating microvesicles of PAH patients and rat models found that the downregulation of miR-150 correlates with the severity and prognosis of PAH (Table 1).\textsuperscript{[135]}

MiR-21 is consistently downregulated in monocrotaline-injected rats but not during hypoxia exposure.\textsuperscript{[132]} MiR-21 expression is induced by pro-inflammatory cytokines such as IL-6\textsuperscript{[136]} and TGF-β/BMP and it is known to drive SMC differentiation.\textsuperscript{[137]} A recent work from Parikh et al. demonstrated on pulmonary artery endothelial cells that BMP-dependent signalling activation of miR-21 represses Rho-kinase activation, thus counteracting the Rho signalling in promoting pulmonary vascular pathology.\textsuperscript{[138]} Moreover, miR-21-null mice presented overexpression of RhoB and hyperactivation of Rho-kinase activity accompanied by exaggerated manifestation of PAH.\textsuperscript{[139]} In vitro administration of a miR-21inhbitor in hypoxia-exposed mice reduces pulmonary vascular remodelling and PAH-associated right ventricular hypertrophy.\textsuperscript{[140]}

One of the first studies providing evidences for the involvement of a specific miRNA in the etiology of human PAH focused on the downregulation of miR-204 (Table 1),\textsuperscript{[141]} which had been previously shown to be associated with cell proliferation in cancer cells\textsuperscript{[142]} and epithelial retinal depolarization.\textsuperscript{[143]} In PASMC STAT3 mediates downregulation of miR-204 which enhances
the activity of Src thereby increasing proliferation and resistance to apoptosis.\textsuperscript{[141]}

A connection between PAH insurgence, the TGF-β signalling pathway and miRNAs has been described in a form of heritable PAH that presents a mutated SMAD9 gene (protein Smad8).\textsuperscript{[144,145]} This work demonstrated the role of Smad8 in promoting miRNA expression by the non-canonical BMP pathway. Specifically, two miRNAs have been described to be regulated by the non-canonical BMP pathway, miR-21 and miR-27a, whose expression are reduced in PAEC and PASMC in PAH patients.\textsuperscript{[146]} Moreover, miR-21 expression levels are correlated with cell proliferation in PAEC derived from PAH patients.\textsuperscript{[144]}

In a monocrotaline-induced rat model, miR-17 suppression with anti-miR-17 treatment has been shown to significantly counteract the pathological severity of the disease.\textsuperscript{[146]} In both in vitro and in vitro treatment with anti-miR-17 a significant increasing of the miRNA’s target p21 has been shown to be a central mechanism in the repression of the SMC proliferative feature of PAH.\textsuperscript{[146]} A hypoxia induced miRNA found to be relevant in the pathology of PAH is miR-210, indeed it has been demonstrated that miR-210 expression increased in microarray analysis on human in hypoxic PASMC and in whole lungs of hypoxic mice.\textsuperscript{[147]} The induction of miR-210 is HIF-1α-dependent and triggers anti-apoptotic effects via directly targeting the transcription factor E2F3.\textsuperscript{[147]} Previously work carried out on PAECs showed an additional mechanism of adaptation to hypoxic conditions in which miR-210, by targeting Iron-Sulfur Cluster Assembly Proteins 1/2 (ISCU) represses mitochondrial metabolism, thus facilitating glycolysis.\textsuperscript{[148]}

Additional clues about the involvement of miRNAs in the hypoxia-induced PAH focused on the cytoprotective action of secreted membrane microvesicles, called exosomes, delivered from mesenchymal stem cells (MSC).\textsuperscript{[149]} Several reports showed that treatment with these microvesicles ameliorates symptoms and prevents progression in PAH and vascular injury.\textsuperscript{[150-152]} In one recent report, it was shown that intravenous delivery of mesenchymal stromal cell-derived exosomes (MEX) interferes with early hypoxic signals in the lung by counteracting inflammation.\textsuperscript{[149]} In particular MEX inhibits MCP-1 release and monocyte recruitment together with decreasing STAT3 phosphorylation, which is an important mediator in the inflammatory and pro-proliferative response triggered by hypoxia.\textsuperscript{[153]} Inhibition of STAT3 activation also affects induction of the miR-17/92 cluster which targets BMPR2.\textsuperscript{[131]} Moreover, treatment with exosomes from MSC also interferes with the STAT3 downregulation by miR-204, which inhibits pro-proliferative action of STAT3 in SMC.\textsuperscript{[141]}

Due to the key role of the PASMC hyperproliferative phenotype in PAH, several studies tried to decipher the role played by miRNAs in this context. MiR-206, previously described as tumor suppressor,\textsuperscript{[154]} is downregulated in hypertensive mouse with excessive right ventricular systolic pressure. Its anti proliferative action is mediated by directly targeting Notch3, whose signalling is known to be elevated in PAH.\textsuperscript{[129]} Two miRNAs highly expressed in SMC; miR-145 and miR-143, have been shown to play a pivotal role in the modulation of SMC phenotype. In particular, their expression is transcriptionally activated by both TGF-β and BMP4 and promotes a contractile phenotype in SMC by targeting the Kruppel-like factor-4 (KLF4).\textsuperscript{[155]} Knock-out of both miRNAs in mice leads to a development of thinner medial layer and hypotension.\textsuperscript{[156]} One recent work investigated the effect of miR-145 modulation in the pathogenesis of PAH and demonstrated that its expression is induced by hypoxia and that its expression is elevated in PAH patients.\textsuperscript{[157]} Genetic ablation or inhibition of miR-145 by antisense oligonucleotides in hypoxia-challenged animal exhibits a protective effect against PAH development.\textsuperscript{[157]} Additional studies reported a downregulation of miR-145 during neonatal formation upon rat carotid balloon vascular injury and adenosine overexpression of miR-145 confers a protective role against vessel wall.\textsuperscript{[158]} On the contrary, a different study reported that mice deleted in miR-143 and/or miR-145, develop reduced neointima formation upon carotid artery ligation, thus pointing to a protective role of miR-145 ablation.\textsuperscript{[159]} This phenotype is probably due to a depletion of SMC in the media due to the absence of these important miRNAs during the development.\textsuperscript{[160]} The effects of miR-145 null mice in hypoxia-induced PAH and neointimal formation is likely dependent on regulatory networks influenced by a plethora of miR-145 direct and indirect targets affecting cytoskeleton dynamics and thus cell migration.\textsuperscript{[159]} It is interesting to speculate that the effect of miR-145 on cytoskeleton organization and cell migration is context-dependent and that the effect of its deregulation can be different depending on the state of smooth muscle cell differentiation and on the microenvironment. Detailed analysis of miRNA expression has been performed in PAH patients by isolating plexiform (PLs) and concentric lesions (CLs).\textsuperscript{[161]} This analysis focused on expression of the endothelial-specific miR-126\textsuperscript{[162]} and the SMC-specific miR-145 and miR-143. Expression of miR-143 and miR-145 was significantly lower in PLs compared with CLs and healthy tissue. Decreased expression of miR-143/145 may reflect the more proliferative and undifferentiated phenotype of SMCs present in PLs. On the other hand, the expression level of miR-126 was higher in PLs when compared to CLs, in accordance with the higher abundance of EC in PLs than in PLs.\textsuperscript{[163]} Thus this study provided a specific miRNA signature for different hypertensive pulmonary lesions potentially providing new diagnostic tools.
Acute lung injury and acute respiratory distress syndrome

Acute lung injury (ALI) is a phenotypically variable acute respiratory failure clinically characterized by progressive hypoxemia, reduced lung compliance and intense inflammation that results from either direct injury to the lung (pneumonia, thoracic trauma, smoke-related lung injury) or an indirect insult (pancreatitis). ALI together with its most severe manifestation namely the acute respiratory distress syndrome (ARDS) has a high incidence and high mortality. Cellular characteristics of ALI and ARDS are the loss of alveolar-capillary membrane integrity consisting of a leakage of the endothelium and degradation of the alveolar basement membrane with the consequent extravasation of fluids from the circulation to the inside the small airways.

It is well recognized that only a small proportion of exposed individuals will ultimately develop these syndromes and has been proposed that there is a genetic predisposition to the development of ALI pathology. Recently, efforts in determining the genetic component of ALI/ARDS pathogenesis have been extended to the involvement of miRNAs in this process (Table 1). Several genes involved in the immune response and inflammation, already described to be targeted by miRNAs, have been suggested as candidates in the establishment of ALI. A detailed study investigating changes in miRNA expression upon lung injury has been performed using the model of injury triggered by high volume tidal ventilation (HVT). In this study HVT applied to mice induced an upregulation of several miRNAs, with a few exceptions. Inflammatory and TGF-β-signalling miRNA-gene networks have been identified by in-silico pathway analysis as significant modulated in the onset of ALI. MiR-21 was one of the most upregulated and treatment of mice with antisense oligonucleotides against miR-21 before HVT ameliorated induction of pulmonary edema associated with the ventilation injury. Other miRNAs associated with inflammation, such as miR-155, let-7 and miR-146, are similarly deregulated upon HVT treatment (Table 1). Another expression analysis on the effects of stress ventilation was performed by stretching rat alveolar epithelial cells to mimic mechanical ventilation. In this study, the majority of miRNAs were again found to be upregulated. Interestingly the highest fold-change occurred in the detection of miR-32*, suggesting a functional role for this miRNA passenger strand. The authors of this work have also demonstrated a functional link between the levels of miR-466d-5p and miR-466f-3p, and the inhibition of stretch-induced permeability.

An analysis of the miRNAs expression profile of mice with bleomycin-induced lung injury showed that miR-127 is down modulated in macrophages. MiR-127 targets the Fc receptor for IgG (IgG FcyRI - CD64) that activate the cells in the clearance of the immunocomplexes and in promoting inflammation by secreting cytokines. The same study demonstrated that overexpression of this miRNA can prevent exaggerated inflammation during injurious stimuli and the set of ALI in mice treated with bleomycin.

A study on the role of miRNAs in the pathogenesis of ALI also identified the involvement of miR-16 in the resolution of pulmonary edema. In this work, mice exposed to hyperoxia exhibited a decrease in miR-16 together with decreased expression of the epithelial sodium channel which is critical for driving fluids out of alveolar space. MiR-16 has been found to target the serotonin transporter (SERT). In vitro experiments using human alveolar epithelial cells demonstrates that overexpression of miR-16 reduces SERT and increases the epithelial sodium transporter proposing miR-16 as a candidate for the treatment of pulmonary edema and prevention of ALI.

miRNAs AS THERAPEUTIC TARGETS

The recent achievements in manipulating miRNA expression in animal models of related human diseases, together with the increasing rate of discoveries about the role played by miRNAs in several human disorders have raised the possibility of using these molecules as a new class of therapeutic tools. Along with this concept, from the gain- and loss-of function studies it appears that dysregulation of miRNAs is well-tolerated in normal tissues but that it can influence the behaviour of the tissues experiencing pathological conditions. The first clinical trial of a miR-122 mimicry as a therapy for suppression of hepatitis C virus (HCV) is currently under going.

Several molecular strategies have been employed in order to down modulate or overexpress miRNAs in vitro. Anti-microRNA oligonucleotides (anti-miR) have been generated and extensively chemically modified in order to increase the effectiveness and stability. Modification of the phosphodiester backbone with a phosphorothioate linkage between nucleotides or including a 2′O-methyl group are two common modifications for increasing stability and decreasing the in vitro clearance. Additionally, antagonirs containing a cholesterol moiety are thought to favour cellular uptake. Special interest has been focused on the use of the Locked Nucleic Acid (LNA) that present a modification in the ribose ring improving nuclease resistant and conferring an high affinity for complementary sequence. LNA antagonirs against miR-33 showed beneficial effects in non-human primates for the treatment of atherosclerosis by increasing the level of high-density lipoprotein and lowering the level of low-density lipoprotein. Systemic delivery of antagonir has been
proven effective in the treatment of cardiac hypertrophy and fibrosis by down modulating miR-133 \cite{182} and miR-21 \cite{183} respectively. The anti-miR-21 therapeutic effect against fibrosis has also proven effective in the lungs \cite{21} and for the treatment of diabetic nephropathy using a mouse model of type 2 diabetes.\cite{183} Currently, the most effective means of delivering anti- or mimic miRNA is through adenovirus-associated virus (AAV).\cite{184} This system benefits from the advantage of potential tissue enrichment as a result of the natural tropism of the different AAV serotypes. Moreover, the exogenous sequence can be continually expressed, resulting in a robust replacement or inhibition of miRNA expression.

It has been demonstrated that the introduction of miR-29 in the lung protects animals from the insurgence of the pathology in bleomycin-treated rats.\cite{185} The therapeutic potential of miR-29 in pulmonary fibrosis was examined in both the induction phase and during established lung disease induced by bleomycin using the Sleeping Beauty (SB) transposon-mediated gene transfer system. Progresses in delivery of SB transposons have been achieved in the treatment of PAH, in inhibiting lung allograft fibrosis,\cite{186} in lymphoma \cite{187} and in various systemic disease, such as haemophilia.\cite{188}

There are a few potential challenges for the clinical application of miRNA modulation methods, which include off-target effects of antagonirs, and the potential toxicity of phosphorothionate oligonucleotides.\cite{189,190} For the treatment of pulmonary diseases, there is the great advantage that miRNA-related therapeutics can be delivered via inhalation. Inhalation will decrease systemic exposure of miRNA-related therapeutics and, therefore, limit potential off-target effects and toxicity to other organs.

**CONCLUDING REMARKS**

miRNA biology is a key mechanism for mediating cellular responses under various stress conditions. Lung tissues are constantly under threat of various stresses, such as chemical irritants and air pollutants. Indeed, growing evidence supports the idea that deregulation of protein coding gene expression as a result of aberrant expression of miRNAs is contributing to various stages of the pathogenesis of pulmonary diseases. Therefore, better understanding of the relationship between miRNAs and their target genes will lead to a development of more effective therapies. It is plausible to apply miRNA mimicry or antagonist as novel therapeutic tools. Finally, it is clear that a characteristic miRNA expression profile (or signature) is linked to a specific pathological condition (Table 1). Thus, miRNA signature can be used as a diagnostic tool or for the development of more personalized therapeutic strategy.

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