MicroRNAs in pulmonary arterial hypertension: pathogenesis, diagnosis and treatment

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Abstract

Pulmonary arterial hypertension (PAH) is a severe and increasingly prevalent disease, manifested by the maladaptation of pulmonary vasculature, which consequently leads to right heart failure and possibly even death. The development of PAH is characterized by specific functional as well as structural changes, primarily associated with the aberrant function of the pulmonary artery endothelial cells, smooth muscle cells, and vascular fibroblasts. MicroRNAs constitute a class of small ≈22–nucleotides–long non–coding RNAs that post–transcriptionally regulate gene expression and that may lead to significant cell proteome changes. While the involvement of miRNAs in the development of various diseases—especially cancer—has been reported, numerous miRNAs have also been associated with PAH onset, progression, or treatment responsiveness. This review focuses on the role of microRNAs in the development of PAH as well as on their potential use as biomarkers and therapeutic tools in both experimental PAH models and in humans. Special attention is given to the roles of miR–21, miR–27a, the miR–17–92 cluster, miR–124, miR–138, the miR–143/145 cluster, miR–150, miR–190, miR–204, miR–206, miR–210, miR–328, and the miR–424/503 cluster, specifically with the objective of providing greater insight into the pervasive roles of miRNAs in the pathogenesis of this deadly condition. J Am Soc Hypertens 2015;9(3):221–234.

Keywords: Hypoxia; lungs; non-coding RNAs; vascular remodeling.

Introduction

Pulmonary arterial hypertension (PAH) is an often fatal and increasingly prevalent disease, characterized by a maladaptive elevation of pressure in the pulmonary vasculature, leading to increased pulmonary arterial pressure and generally resulting in right–sided heart failure and eventually death.1 The currently available Dana Point classification divides pulmonary hypertension into five distinct subtypes based on their general pathophysiology and treatment responsiveness: (1) PAH; (2) pulmonary hypertension (PH) due to left heart disease; (3) PH due to interstitial lung diseases and/or hypoxia (PHILD); (4) chronic thromboembolic PH (CTEPH); and (5) PH with different mechanisms2; it is thus quite clear that the clinically presenting PH phenotype may result from different pathophysiological processes. This review focuses mainly on PAH, even though it represents no more than approximately 10% of the total PH population; however, with respect to its severity, poor prognosis, and generally poor treatment responsiveness, it has attracted a great deal of attention even within the microRNA field.

In a broad medical context, PAH should be perceived as a microvascular and angioproliferative disease affecting lung vasculature. From the perspective of the pathophysiology of the disease, the PAH paradigm is based on genetic predisposition (mutations in bone morphogenetic protein [BMP] receptor type 2 [BMPR2])3–5, inflammation, vascular tone imbalance, vessel injury, increased pulmonary artery endothelial cells (PAECs) and smooth muscle cells (PASMCs) proliferation and resistance to apoptosis, increased proliferation of fibroblasts in the adventitial layer of the vascular wall, and the presence of in situ thrombosis.6–8

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The typical morphologic features of PAH include the thickening of the pulmonary adventitia and venous hypertrophy (Figure 1). In immunohistochemical studies, the increased expression of transforming growth factor (TGF)–β and matrix proteins (elastin, fibronectin, tenascin–C, and glycosaminoglycans) has been identified within the vessel wall.6,8 All of these contributing factors lead to severe remodeling, which subsequently causes a clustered appearance, resulting in the formation of complex concentric andplexiform lesions typical of the disease and leading to the occlusion of blood vessels.9,10

While the development of PAH is clearly the result of complex interplay between PAECs, PASMCs, fibroblasts, and blood cells, therapeutic interventions affecting these complex processes have not yet fulfilled established objectives. Currently available PAH therapies are limited and although they do provide a survival benefit, they are not curative and PAH remains associated with a poor long-term prognosis.11 Novel therapeutic and diagnostic approaches are thus urgently needed.14

In order to better understand PAH pathogenesis, a variety of animal models has been developed to simulate the situation occurring in affected individuals, while also doubling as models for preclinical drug testing and design.12,13 A large number of various PAH models reflect the equally large number of the various pathophysiological pathways involved in PAH development. The most widely used chronic hypoxia exposure model is based on the phenomenon of hypoxic pulmonary vasoconstriction, which occurs in lung vasculature during repeated or chronic hypoxia exposure. Vessel vasoconstriction subsequently leads to increased pulmonary pressure, arterial remodeling, and PAH. It has been shown that the addition of SU5416, an agent which blocks vascular endothelial growth factor (VEGF) receptors, leads to faster PAH progression; combinations of SU5416 and chronic hypoxia are also one of the most commonly used models.12,13 With the beginning of the gene knock–out/knock–in era, new models were based on the silencing or promotion of the expression of crucial genes involved in PAH pathogenesis (eg BMPR2,14 5-lipoxygenase,15 or adenosine pathways16); a detailed overview of these models is provided in a review by Gomez–Arroyo.12 In addition to the above–mentioned models, the monocrotaline exposure model of PAH is still being used for PAH studies; this involves the application of macrocyclic pyrrolizidine alkaloid monocrotaline, which affects the function of PAECs (and other cells), thereby leading to pulmonary vasculature remodeling and PAH development.17

Recent advances in the study of microRNAs (miRNAs, miRs) indicate that they significantly affect regulatory feedback loops involved in the pathophysiological processes subsequently leading to PAH (for example, endothelial dysfunction, PASMC proliferation, or arterial remodeling); a study of miRNAs in PAH may thus reveal new pathways and connections not described to date. This review thus specifically focuses on their potential pathogenic, diagnostic, and even therapeutic roles in the area of PAH.

**MicroRNAs: Biogenesis and Function**

miRNAs are small non–coding RNAs that regulate the expression of target protein–coding genes by promoting
the degradation or suppressing the translation of target mRNAs. miRNA biogenesis as well as the mechanisms of miRNA–induced gene silencing have already been well described, and it appears that the basic steps are highly conserved among various species; an overview of the current understanding of the biogenesis of miRNAs is provided in Figure 2.

In general, miRNA biogenesis begins with the transcription of a miRNA gene, typically by RNA polymerase II/III, which subsequently generates a long primary (pri)–miRNA transcript containing typical, short internal stem–loop structures. The Drosha enzyme complex guided by the dsRNA–binding protein DGCR8 processes the pri–miRNA transcript into a 60–100 nt hairpin structure called precursor (pre)–miRNA in the nucleus. The released stem–loop structure is exported from the nucleus by Exportin–5/RanGTP; the pre–miRNA molecules then undergo Dicer–catalyzed processing, which is a process that gives rise to a 22 nt double–stranded (ds)–RNA product containing the mature miRNA guide (miRNA–5p) strand and the passenger (miRNA–3p or miRNA*) strand.

Gene silencing mediated by mature miRNAs is the result of several distinct mechanisms that require the mature miRNA to form a complex with several proteins, including Argonaut (Ago) family members Ago–1 and Ago–2, that is the two most highly expressed Ago proteins present in mammalian cells. The mature miRNA loaded in the Ago protein is then called an RNA–induced silencing complex (RISC), which mediates post–transcriptional silencing by reducing mRNA stability or by translational blocking, depending on the degree of complementarity of the miRNA seed sequence (nucleotides 2–8) with specific regions in the 3′–untranslated region (3′–UTR) of the target genes.

When complementarity between mRNA and miRNA is complete, the mRNA is cleaved. When complementarity is imperfect, a variety of the miRNA/mRNA/RISC complexes may cause premature termination or induce ribosome dissociation. Deadenylation, decapping, and exonuclease action results in miRNA degradation.

**miRNA Biogenesis and PAH Development**

The above–mentioned processing steps are necessary for proper smooth muscle development, based on the studies of smooth–muscle restricted knockout of Dicer in mice. Dicer knockout results in the inhibition of blood vessel maturation, which is of particular importance in PAH development. Moreover, in an attempt to identify miRNAs associated with PAH, Caruso et al established that Dicer levels are significantly reduced in both monocrotaline and chronic hypoxia exposition animal models during the progression of PAH. The levels of other critical enzymes (Drosha, exportin–5) were measured, and Drosha was also shown to be downregulated, albeit only on day 2 in the hypoxic model and on day 21 in the monocrotaline model. These results make the evaluation of miRNA levels in PAH more complex and may be also one of the reasons for the variability of results in various studies, described in greater detail further on.

**Individual miRNAs Involved in PAH Pathogenesis**

The following section provides an overview of currently described miRNAs associated with PAH. For the sake of clarity, miRNAs are listed according to their numbers, not according to year of discovery or relevance. The information listed here is also summarized in Table 1 and Figure 3.
miR–21

Common changes in the transcriptome of the hypoxic cells include the upregulation of hypoxia–inducible factor 1α (HIF–1α), along with the altered expression of a number of mitogenic as well as vasoactive molecules, cytokines, and growth factors. Levels of miR–21 have been reported to be both downregulated and upregulated under hypoxic conditions, thus indicating a conflicting but likely important role of this miRNA in PAH development.

Table 1

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Target mRNA/Signaling Pathway</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR–21</td>
<td>PPARα, Sprouty–2, BMPR2, PDCD4 Rho–kinase signaling</td>
<td>MiR–21 affects SMCs phenotypic switch, increases proliferation and decreases apoptosis.</td>
<td>32–35</td>
</tr>
<tr>
<td>miR–27a</td>
<td>PPARγ</td>
<td>Via autoregulatory feedback loop with PPARγ, miR–27a upregulation leads to increased cellular proliferation.</td>
<td>36</td>
</tr>
<tr>
<td>miR–17–92</td>
<td>HIF–1α, BMPR2, E2F1 STAT3–miR–17/92–BMPR2 pathway</td>
<td>c–myc, IL–6/STAT3 and p53 affects miR–17–92 cluster expression, that is increased in PAH resulting in increased proliferation and decreased apoptosis.</td>
<td>37–43</td>
</tr>
<tr>
<td>miR–124</td>
<td>NFAT, MCP–1, PTBP1 Notch1/PTEN/FOXO3/p21Cip1 and p27Kip1 signaling</td>
<td>miR–124 is involved in the development of proliferative, migratory, and inflammatory phenotype of PASMCs.</td>
<td>44.45</td>
</tr>
<tr>
<td>miR–138</td>
<td>Mst1, S100A1</td>
<td>miR–138 causes resistance to apoptosis and its upregulation leads to endothelial dysfunction and vasoconstriction.</td>
<td>46–50</td>
</tr>
<tr>
<td>miR–143/145</td>
<td>KLF4, KLF5, myocardin</td>
<td>miR–143/145 cluster affect SMCs phenotypic switch. Expression of this cluster is higher in concentric lesions (compared with plexiform lesions).</td>
<td>51–53</td>
</tr>
<tr>
<td>miR–150</td>
<td>—</td>
<td>Reduced miR–150 levels associated with poor survival in PAH</td>
<td>54</td>
</tr>
<tr>
<td>miR–190</td>
<td>KCNQ5</td>
<td>miR–190 affects vascular tone and calcium influx into SMCs via targeting K+–channel KCNQ5.</td>
<td>48,55,56</td>
</tr>
<tr>
<td>miR–204</td>
<td>STAT3, SHP/Src pathway</td>
<td>miR–204 is downregulated under hypoxic conditions, which leads to activation of STAT3 pathway promoting proliferation and PAH progression.</td>
<td>57</td>
</tr>
<tr>
<td>miR–206</td>
<td>Notch3, HIF–1α</td>
<td>miR–206 is decreased under hypoxic conditions and via Notch3 and HIF–1α pathways it affects PAH development.</td>
<td>58–59</td>
</tr>
<tr>
<td>miR–210</td>
<td>E2F3</td>
<td>miR–210 has an antiapoptotic effect on PASMCs during hypoxia.</td>
<td>60</td>
</tr>
<tr>
<td>miR–328</td>
<td>IGF1, LTCC–α1C</td>
<td>miR–328 affects PAH via promoting proliferation (IGF1 signaling) and vasoconstriction (LTCC–α1c downregulation).</td>
<td>61</td>
</tr>
<tr>
<td>miR–424/503</td>
<td>FGF2, FGF1R Apelin signaling</td>
<td>miR–424/503 are downregulated in PAH as well as apelin. Restoration of apelin and miR–424/503 levels reduces PAH severity.</td>
<td>62–64</td>
</tr>
</tbody>
</table>

BMP, bone morphogenetic protein; BMPR2, BMP receptor type 2; E2F3, transcription factor E2F3; E2F1, transcription factor E2F1; FGF1, fibroblast growth factor 1 receptor; FGF2, fibroblast growth factor 2; HIF–1α, hypoxia–inducible factor 1α; IGF1, insulin growth factor 1; KCNQ5, Potassium voltage-gated channel subfamily KQT member 5 protein; KLF4, Kruppel-like factor 4; KLF5, Kruppel-like factor 5; LTCC–α1C, L-type calcium channel subunit alpha1C; MCP–1, monocyte chemotactic protein–1; miRNAs, microRNAs; MiRs, microRNAs; Mst1, human macrophage stimulating protein 1; NFAT, nuclear factor of activated T–cells; Notch 3, Neurogenic locus notch homolog 3 protein 3; PAH, pulmonary arterial hypertension; PASMCs, pulmonary artery smooth muscle cells; PDCD4, programmed cell death protein 4; PPARα, peroxisome proliferator–activated receptor–α; PTBP1, polypyrimidine tract–binding protein 1; S100A1, S100 calcium-binding protein A1; SHP, Src homology region 2 domain–containing phosphatase; SMC, smooth muscle cells; SPRY2, Sprouty homolog 2; Src, src kinase; STAT3, Signal transducer and activator of transcription 3.

miR–21
of miR–21 levels is likely driven by both HIF–dependent and HIF–independent mechanisms (eg, the induction of Akt2 activity),68,69 which may be one of the reasons behind the conflicting results of the studies mentioned here. Additional reasons likely include variations in utilized experimental models and conditions. A study by Caruso et al reports miR–21 downregulation in the monocrotaline animal model but not in the chronic hypoxia model,32 while Yang et al demonstrated miR–21 upregulation in the chronic hypoxia model as well.35 While Caruso et al treated PASMCs with TGF–b1 and BMP4, thus inducing the downregulation of miR–21,32 Sarkar et al and Yang et al cultured PASMCs under hypoxic conditions, resulting in the upregulation of miR–21.34,35; similar results were also obtained when PAECs were cultured under hypoxic conditions.33 The consequences of miR–21 upregulation are dealt with in greater detail further on in this text, especially as this is a frequently reported phenomenon.

The overexpression of miR–21 in the PASMC causes PASMCs to switch from a quiescent phenotype to a proliferative phenotype, which may be reversed following an anti–miR21 application.34,35 miR–21 has plentiful target genes—mostly tumor suppressors—which, upon repression by miR–21, derepress proliferation and suppress apoptosis. These targets include, for example, programmed cell death protein 4 (PDCD4),70 Sprouty 2 (SPRY2), and peroxisome proliferator–activated receptor–α (PPARα).71,72 Other targets include proteins integral to BMP, BMPR2, and Rho/Rho–kinase signaling, thus connecting miR–21 to hypoxia, inflammation, and angiogenesis signaling pathways, as shown in a network bioinformatics analysis by Parikh et al.33 The authors confirmed their predictions in PAECs, showing that miR–21 is upregulated under hypoxic conditions and following BMPR2 administration and that one of the effects of miR–21 is the inhibition of RhoB expression and Rho–kinase activity, in turn leading to molecular changes consistent with suppressed angiogenesis and vasodilation.33 Yang et al further added a negative feedback loop to the described pathways by demonstrating that upregulated miR–21 (both in hypoxic mouse models and human PASMCs exposed to hypoxia) results in the downregulation of BMPR2.35

Last but not least, miR–21 upregulation was reported not only in the above described experiments—connecting miR–21 to the initiation of PAH—but also in samples of severe plexiform vasculopathy, as seen in severe PAH.51 A study by Bockmeyer et al compares concentric lesions and plexiform lesions and shows the upregulation of miR–21 and miR–126; that is, miRNA specific to endothelial cells, in plexiform lesions, thus indicating a more prominent angiogenic phenotype and enhanced cell proliferation in these lesions.51

Taking into account the above–mentioned facts, along with the finding that miR–21 is a known shear–stress responsive miRNA73 and that its elevated levels are associated with fibrosis,74,75 miR–21 clearly deserves further research attention designed to reveal its true potential in PAH development.

miR–27a

miR–27a is one of the miRNAs upregulated due to hypoxia in experimental animals.36 The upregulation of miR–27a is accompanied by PPARγ downregulation and the proliferation of PAECs. Interestingly, PPARγ is not only a target of miR–27a but also regulates miR–27a
expression along with other transcription factors (SP1 and EGR1), thereby creating a regulatory feedback loop. The therapeutic restoration of PPARγ levels (eg, via miR–27a inhibitors) may thus represent a new approach to affecting PAECs proliferation.

miR–17–92 Cluster

The miR–17–92 cluster is one of the best–characterized miRNA families controlling cell development, apoptosis, and proliferation, whose genomic amplification or aberrant elevation are frequently observed in a variety of tumor types. This cluster consists of six distinct miRNAs (miR–17, miR–18a, miR–19a, miR–20a, miR–19b–1, and miR–92–1), each of which have a specific set of target genes that exert their functions.

With respect to hypoxia and PAH, it has been reported that miR–17–92 cluster expression is regulated negatively by p53 and positively by the c–myc transcription factor, and the IL–6/STAT3 pathway, consequently resulting in an increase or decrease, respectively, of HIF1α or BMPR2; that is, two miR–17–92 cluster targets. Furthermore, miR–17 expression was shown to be regulated in a c–myc and p53 independent fashion via arginase II.

A p53–mediated decrease in miR–17–92 is believed to be part of a complex process of hypoxia–mediated apoptosis; the inhibition of miR–17 and miR–20a also leads to pronounced cell apoptosis under hypoxic conditions. The induction of the miR–17–92 cluster via c–myc leads to the inhibition of transcription factor E2F1, which induces c–myc and miR–17–92 expression in a positive feedback loop, thus promoting proliferation and suppressing apoptosis of various cell types. Last but not least, increased miR–17–92 expression via IL–6/STAT3 leading to BMPR2 downregulation has been proposed, due to the fact that IL–6 levels, as one the factors associated with PAH development, are increased and BMPR2 levels are decreased in patients with PAH.

From a comprehensive point of view, the above mentioned studies clearly indicate that miR–17–92 cluster upregulation results in reduced apoptosis (especially in resistance to hypoxia–induced apoptosis) and increased proliferation, two phenomena regularly reported to occur during PAH pathogenesis. As the siRNA inhibition of STAT3 resulted in a reduction of PAH severity and anti–miR–17 targeting of miR–17 resulted in decreased right ventricle systolic pressure and pulmonary vascular remodeling, the therapeutic targeting of the miR–17–92 cluster may constitute an innovative approach to future PAH treatment.

miR–124

In connection with PAH, miR–124 was introduced as an anti–proliferative miRNA, downregulated under hypoxic conditions in PASMCs and in the human vascular fibroblast. In a study by Kang et al, miR–124 was reported to suppress the transactivation of the nuclear factor of activated T–cells (NFAT) by targeting multiple genes and thus inhibiting the proliferation of PASMCs. This study was based on the assumption that the NFAT signaling pathway is linked to PASMC proliferation leading to PAH. Considering the downregulation of miR–124 occurring in hypoxia–treated PASMC, it is possible that miR–124 acts as an anti–proliferative and pro–differentiation miRNA; the potential therapeutic restoration of miR–124 levels in the treatment of PAH may thus be suggested.

A study by Wang et al indicates that miR–124 controls the proliferative, migratory, and inflammatory phenotypes of another cell type involved in PAH: pulmonary vascular fibroblasts. The transfection of vascular fibroblasts with miR–124 mimic led to a significant reduction in proliferation, migration, and monocyte chemotactic protein–1 (MCP–1) expression. On the other hand, anti–miR–124 treatment had the opposite effects, including, for example, increased proliferation, migration, and increased MCP–1 expression. Another putative target of miR–124 was detected and identified as the alternative splicing factor polypyrimidine tract–binding protein 1 (PTBP1). PTBP1 is upregulated both in vivo and in vitro in bovine as well as human pulmonary artery fibroblasts and miR–124 binding to its 3’UTR affects the regulation of Notch1/PTEN/FOXO3/p21Cip1 and p27Kip1 signaling. This study also showed that miR–124 levels are affected due to altered histone acetylation; as a result, therapies directed at restoring miR–124 function—including histone deacetylase (HDAC) inhibitors—constitute attractive options for the possible treatment of PAH.

miR–138

miR–138 has been reported as affecting HIF–1α expression in various types of tumors, thus suppressing their invasion and metastasis. Recently, it has also been associated with hypoxic pulmonary artery remodeling and hypoxia–induced endothelial cell dysfunction. A study conducted by Li et al was based on the hypothesis that unbalanced apoptosis is a major cause of structural remodeling of vasculature associated with PAH. An increased expression of miR–138 in the PASMCs was shown to be hypoxia–dependent and miR–138 was shown to target proteins of Bcl–2 and Akt pathways (with the serine/threonine kinase Mst1 being the predicted target), thus mediating its antiapoptotic effects.

In the vasculature of the lower extremities, miR–138 was shown to target the S100A1 protein (calcium sensor), thus affecting the production of nitric oxide (NO) and leading to endothelial dysfunction with pronounced vasoconstriction. Moreover, this overexpression may be caused by proinflammatory molecules, such as endothelin–1, TNF, and angiotensin II. The reported mechanisms may be theoretically applicable to PAH (according to studies
performed with miR–143 and miR–145; see below for details), and the therapeutic silencing of miR–138 may be beneficial for patients with PAH.

**miR–143/145 Cluster**

miR–145 is typically co-expressed together with miR–143; both miRNAs are involved in the regulation of the vascular SMC phenotype. As this cluster is highly expressed in SMC, its vascular functions have been examined in great detail in numerous studies. It has been shown that the deregulation of miR–143/145 leads to a phenotypic switch of SMCs (from quiescent phenotype to proliferative phenotype and vice versa), which is connected with vascular injury and may contribute to the development of PAH.

The regulation of miR–143/145 cluster expression is complex; as such it is ensured by means of various stimuli including the serum response factor (SRF), Krüppel-like factor 2 (KLF2), and CBF1. The promoter region of the miR–143/145 cluster has been found to include the CArG box while SRF was shown to influence miR–143/145 expression by CArG box binding. The binding of SRF is further promoted by SRF cofactors, that is myocardin and myocardin–related transcription factors (MRTF), with myocardin being upregulated by TGFβ and MRTF being upregulated by BMP4. An increase in the levels of any of these signaling molecules leads to an increased expression of miR–143/145, also reported in the case of PAH.

Upon upregulation, these miRNAs directly repress a related group of multiple target genes, two of them being KLF4 and KLF5. The downregulation of these two targets is associated with an increase in SMC specific genes (smooth muscle actin, calponin, etc), which is, in turn, associated with promoted differentiation and decreased proliferation of SMCs. KLF4 further inhibits the expression of myocardin and MRTF, and the inhibition of KLF4 by miR–143/145 leads to an increase in myocardin and MRTF, thus promoting miR–143/145 expression. Myocardin itself constitutes another putative target of miR–143/145, thus adding another regulatory feedback loop to the above described network.

When focusing specifically on PAH, an increase in miR–143/145 expression was reported in PAH patients’ pulmonary arteries (and localized within SMCs), in primary PASMCs cultured from patients with a known BMPR2 mutation and in BMPR2 R899X knock–in mice. On the other hand, using miR–145 knockout mice and mice treated with anti–miR–145 (but not with miR–143), Caruso et al demonstrated the protective effect of miR–145 downregulation on PAH development. According to the previously reported studies and based on the fact that miR–143/145 expression is higher in concentric lesions compared with plexiform lesions, it is possible to hypothesize that miR–143/145 upregulation is associated with increased “muscularization” of PAH vessels and that relative down-regulation (as observed in plexiform lesions) is connected more to the angioproliferative phenotype associated with PAECs proliferation. Both of these conditions would then increase blood pressure in pulmonary circulation leading to PAH. Given the above described effects of miR–21 and miR–126, it is clear that more miRNAs are involved in the SMCs phenotypic switch and that more complex studies are needed in order to establish the true potential of the above-mentioned miRNAs in PAH development.

**miR–150**

miR–150 is the first circulating miRNA to be suggested as an independent predictor of poor survival in a small cohort of PAH patients. Following miRNA profiling, 58 miRNAs were identified as being altered in plasma collected from PAH patients, with miR–150 being the most downregulated one. Subsequent experiments indicated that its levels are also decreased in the lungs of monocrotaline–treated animals, suggesting that this miRNA may play a diagnostic as well as a pathogenetic role. However, Rhodes’ results must be verified on a larger population cohort in order to examine the possible involvement of miR–150 in the development of PAH, especially as this miRNA affects the expression of p53 and the production of lung surfactant.

**miR–190**

miR–190 has been shown to be upregulated in a study by Lin et al; however, this study primarily focused on the previously discussed miR–138. In a follow-up work, Lin et al showed that the upregulation of miR–190 induced by hypoxia leads to the downregulation of the voltage–gated K⁩ channel subfamily member KCNQ5 (Kv7.5). This channel was previously established as important for Ca²⁺ ion influx and the regulation of vascular tone; the upregulation of miR–190 thus leads to membrane depolarization (via decrease in Kv7.5), causing Ca²⁺ influx and resulting in profound vasoconstriction, which is one of the pathophysiological features of PAH.

**miR–204**

miR–204 constitutes one of the first miRNAs associated with arterial remodeling; its levels were reported to be downregulated in PAH, both in the lungs of experimental animals and in patient lung samples. Courboulin et al localized the expression of this miRNA to PASMCs, which, due to miR–204 downregulation, resulted in an increase in proliferation and a decrease in apoptosis. Importantly, miR–204 treatment of monocrotaline animals led to a
significant lowering of blood pressure in the pulmonary artery and media thickness.\textsuperscript{57}

The molecular mechanisms underlying the above described mechanisms are probably the outcome of STAT3 signaling deregulation. Contrary to the miR–17–92 cluster (with levels upregulated due to STAT3 activation during the initiation of PAH), the levels of miR–204 are downregulated. This downregulation in turn leads to STAT3 activation by means of SHP2–Src signaling. This positive feedback loop, along with miR–17–92 activation, thus probably contributes to the acceleration of PAH development.

\textit{miR–206}

miR–206 constitutes another candidate miRNA involved in PAH pathogenesis, especially thanks to its effects on proliferation, apoptosis,\textsuperscript{58,59} and HIF–1\(\alpha\) expression.\textsuperscript{59} The downregulation of this miRNA is generally associated with a more aggressive phenotype of various cancers due to increased proliferation and angiogenesis; on the other hand, the application of miR–206 in various cell cultures generally leads to cellular differentiation and repression of proliferation.\textsuperscript{58}

In recently conducted studies focusing on the role of miR–206 in PAH, the downregulation of miR–206 under hypoxic conditions was reported both in animal models as well as in cultured PASMCs.\textsuperscript{58,59} However, conflicting results were obtained when miR–206 transfection was performed. Jalili et al observed an increased differentiation and a block in proliferation and migration of PASMCs, likely due to miR–206 downregulation of Notch3.\textsuperscript{58} Yue et al reported surprising results, as miR–206 transfection led to a paradoxic increase in HIF–1\(\alpha\) (miR–206 target) levels, promoting PASMC proliferation by modulating the HIF–1\(\alpha\) Fhl–1 pathway.\textsuperscript{58} The paradoxic upregulation of HIF–1\(\alpha\) levels was hypothesized to be a result of the endogenous competition of other miRNAs with miR–206 for miRNA–processing enzymes and the HIF–1\(\alpha\) miRNA–response element in its 3′UTR; the possibility that the miR–206 effect is quantity–dependent was also suggested.\textsuperscript{59} In view of the conflicting results obtained from the above–mentioned studies, additional work is clearly needed in order to shed more light onto the proper function of miR–206 in PAH development.

\textit{miR–210}

miR–210 is a typical miRNA regulated by hypoxia, that is the so–called hypoxamir.\textsuperscript{89} The upregulation of miR–210 under hypoxic conditions has been shown to affect the regulation of cell growth, angiogenesis, and apoptosis, and also the mitochondrial metabolism in various human tumor models.\textsuperscript{89,90} Since hypoxia acts as an important stimulus for human PASMC proliferation and PAH development, Gou et al performed miRNA microarray assays in hypoxia–treated and control PASMCs.\textsuperscript{90} As expected, hypoxia caused an upregulation of miR–210. The authors reported this upregulation to be HIF–1\(\alpha\)–dependent and identified E2F3 as a miR–210 target responsible for the reduced apoptosis seen in the hypoxia–induced PASMCs. The inhibition of miR–210–induced apoptosis in PASMCs constitutes an important finding, suggesting a potential role for miR–210 restoration as a therapy for PAH.\textsuperscript{60}

\textit{miR–328}

A study by Guo et al demonstrated that miR–328—a miRNA primarily localized within the PASMCs—is downregulated in the pulmonary artery of experimental animals under hypoxic conditions.\textsuperscript{61} miR–328 inhibited L–type calcium channel–\(\alpha\)1C expression through its binding site localized within its 3′–UTR, and Guo et al report that this inhibition attenuates the pulmonary arterial vasoconstrictive response.\textsuperscript{61} Moreover, the inhibition of another target, that is insulin growth factor 1 receptor (IGF1R), led to the apoptosis of PASMCs.\textsuperscript{61} Therefore, it seems that miR–328 influences both the vasoactive response of the vascular wall as well as the apoptotic escape of the PASMCs, though the exact molecules and targets involved in the apoptosis response via miR–328 remain in need of further clarification.

\textit{miR–424/503 and Apelin Signaling}

The involvement of apelin (APLN) –apelin receptor (APLNR) signaling axis in the development of PAH has recently been reviewed.\textsuperscript{62} APLN is a highly remarkable peptide which is expressed in the ECs of both systemic and pulmonary vasculature; its expression is regulated by HIF–1\(\alpha\) and BMPR2: that is the two key players in PAH.\textsuperscript{62} Apart from its role in angiogenesis, APLN regulates ECs and SMCs apoptosis and proliferation complementary and opposite to VEGF.\textsuperscript{62,63}

In 2013, Kim et al suggested that a miRNA–dependent association exists between APLN and fibroblast growth factor 2 (FGF2) signaling in PAECs.\textsuperscript{63} APLN deficiency in these cells causes an increased expression of FGF2 and its receptor (FGFR1) as a result of the decreased expression of miR–424 and miR–503, both of which directly target FGF2 and FGFR1 3′UTRs. Moreover, the authors showed that miR–424 and miR–503 were downregulated in PAH.\textsuperscript{63} The overexpression of these two miRNAs exerted antiproliferative effects in both normal and PAH PAECs and inhibited the induction of proliferation in PASMCs based on use of the PAEC–conditioned medium.\textsuperscript{63} This is consistent with findings established by Alastalo et al, who report that the administration of apelin reversed PAH in mice, which suggests that apelin may be potentially...
effective in treating PAH by rescuing BMPR2 and PAEC dysfunction.64

Interestingly, both APLN91 and miR–424–50392 genes are located on chromosome X. Since PAH is reported to occur more often in women than in men (>2:1),93 this may be one of the factors contributing to observed differences in disease prevalence, along with the previously described effects of estrogens.93

Diagnostic and Therapeutic Difficulties in the Use of MicroRNAs

The numerous studies listed above clearly demonstrate that miRNAs are associated both with the development of PAH and with the progression of the disease. Nevertheless, the clinical utility of these findings currently remains unclear.

When considering the diagnostic potential of miRNAs, the general definition of a good biomarker states that such a biomarker should be easily measured while still reflecting the pathophysiological process itself.94 Originally, it was believed that most miRNAs within the circulation are enclosed in apoptotic bodies,95 microvesicles,96 or exosomes97 and, as such, cannot be used as good biomarkers due to the high instability of isolated microparticles. However, Arroyo et al have demonstrated that almost 90% of circulating miRNAs exist in a stable complex, primarily with the Ago2 protein,98 and Chen et al showed that levels of circulating miRNAs measured in individuals are stable and that the results are repeatable.99 With respect to the specificity of miRNAs in terms of reflecting pathophysiological processes, the profiles of circulating miRNAs were shown as not stochastic and capable of reflecting certain processes, whereas these miRNAs are actively pumped from the cells or released due to cell necrosis or apoptosis.95,100–102

Methodological difficulties associated with the introduction of miRNAs as serum biomarkers for PAH include primarily the following: a lack of standardization, a lack of reference molecules, a lack of longitudinal studies, and a lack of big sample–sized studies.103,104 Results associated with miR–15054 or the recently described miR–26a105 clearly require additional verification using a larger population cohort. A cost–effectiveness analysis should be subsequently performed in order to establish whether the determination of circulating miRNAs is capable of producing a sufficient amount of new information and compensating the costs of utilized methods.

When focusing on the therapeutic potential of miRNAs, two main strategies may be defined using (i) miRNA mimics (substitution therapy in the case of downregulated miRNAs) and (ii) anti–miRs (inhibition therapy in the case of upregulated miRNAs). McDonald et al hypothesize that it may be possible to achieve a wide distribution of miRNAs via systemic approaches (eg, intravenous, subcutaneous, or intraperitoneal routes); however, this approach is likely not suitable, since off–target effects of miRNA modulation may be apparent, especially in the liver, where systematically applied anti–miRs preferentially accumulate.106,107 While the direct targeting of miRNA–based pathways via local delivery to the lung is logically essential in order to minimize possible off–target effects, local delivery to lung vessels represents a huge technological challenge. In a previously mentioned paper, Kim et al report that the intranasal administration of GFP–expressing control lentivirus or lentivirus expressing miR–424, miR–503, and GFP (424/503–GFP) to rat models resulted in a reduction in right ventricular systolic pressure, in a lower expression of proliferating cellular nuclear antigens (PCNA), and in decreased expression of both FGF2 and FGFR1 in 424/503–GFP treatment models. It seems that intranasal application of miRNA mimics could be an attractive therapeutic route of administration and it was proposed that APJ pathway could be an elegant target in the miR–based treatment of PAH, as previously mentioned.108

The other therapeutic approach is based on the delivery of anti–miRs, locked–nucleic acids (LNA), or antagomiRs,109 specifically silencing miRNA expression and leading to the upregulation of hundreds of genes predicted to be repressed by the down–modulated miRNAs.109 Current studies of antimiR application in PAH yielded some promising results—in a recent paper, Pullamsetti et al suggest that A–17 (blocker of miR–17) improves heart and lung function in experimental PAH by interfering with lung vascular and right ventricular remodeling.10 A study by Brock et al reports that the transfection of human pulmonary artery smooth muscle cells (HPASMCs) with antagomiR–20a resulted in the activation of downstream targets of BMPR2 and prevented vascular remodeling in hypoxia–induced pulmonary hypertension.110 Moreover, the proliferation of HPASMCs was found to be reduced upon transfection with antagomiR–20a in these experiments. These studies have shown that antagomiRs action occurs only following repeated application and that the effect is not immediate; a period of time is necessary in order to achieve the expected effect and the optimization and stabilization of the antagomiRs effect represents an important technological challenge for the future.107

In addition to technological difficulties associated with achieving the expected effect on miRNA expression and on the functioning of molecular targets, a number of other general complications may slow down the development of miR–based therapeutics. At present, there is no antidote to miRNA therapy which would allow for an immediate reversal of any potentially undesirable effects.107,110 Moreover, potential toxicity and various interactions of RNA–interference approaches with commonly used drugs (eg, heparin is known to affect circulating miRNA levels111) are currently unknown—any efficacy and safety concerns must therefore be resolved first.
Nevertheless, and in spite of the fact that it is necessary to tackle a range of technological difficulties, the current rapid development of our understanding of the actual biological roles of miRNAs in PAH development as well as partial successes in the field of RNAi therapy clearly justify an optimistic approach to future developments.

References


