MicroRNAs in Pulmonary Arterial Hypertension

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Abstract

Pulmonary arterial hypertension (PAH) is a devastating disease without effective treatment. Despite decades of research and the development of novel treatments, PAH remains a fatal disease, suggesting an urgent need for better understanding of the pathogenesis of PAH. Recent studies suggest that microRNAs (miRNAs) are dysregulated in patients with PAH and in experimental pulmonary hypertension. Furthermore, normalization of a few miRNAs is reported to inhibit experimental pulmonary hypertension. We have reviewed the current knowledge about miRNA biogenesis, miRNA expression pattern, and their roles in regulation of pulmonary artery smooth muscle cells, endothelial cells, and fibroblasts. We have also identified emerging trends in our understanding of the role of miRNAs in the pathogenesis of PAH and propose future studies that might lead to novel therapeutic strategies for the treatment of PAH.

Keywords: pulmonary arterial hypertension; microRNA; pulmonary artery smooth muscle cell; pulmonary artery endothelial cells; fibroblasts

Clinical Relevance

We have reviewed the current knowledge about microRNA (miRNA) biogenesis, miRNA expression pattern, and their roles in regulation of pulmonary artery smooth muscle cells, endothelial cells, and fibroblasts. We have also identified emerging trends in our understanding of the role of miRNAs in the pathogenesis of pulmonary arterial hypertension and propose future studies that might lead to novel therapeutic strategies for the treatment of pulmonary arterial hypertension.

Pulmonary arterial hypertension (PAH) is a fatal disease without effective treatment (1, 2). PAH can be classified into many subcategories, including idiopathic PAH (IPAH), heritable PAH (HAPAH), and PAH associated with other diseases, such as connective tissue diseases (3). All types of PAH share common pathological changes, such as pulmonary artery endothelial cell (PAEC) proliferation; pulmonary artery smooth muscle cell (PASMC) proliferation, migration, and contraction; inflammation; and fibroblast proliferation, activation, and migration. Numerous factors contribute to the pathogenesis of PAH, including genetic,

epigenetic, and environmental factors. MicroRNAs (miRNAs) are small, non-coding endogenous RNA molecules consisting of ~21 to 25 nt (4, 5). A single miRNA can regulate hundreds of genes or proteins and conversely multiple miRNAs can regulate one protein (5). In this review, we summarize recent knowledge about the role of miRNAs in the pathogenesis of PAH, particularly in the three principal cell types involved, namely, PASMCs, PAECs, and fibroblasts (Figure 1), and provide a perspective for needed research in this field. Although we do not include a discussion on the role of miR-126 in PAH, a recent review has summarized

the data reported in the 2013 Grover Conference (6).

miRNA-Mediated Gene Regulation

The generation of mature miRNAs is a multistep process (Figure 2). In the nucleus, miRNA genes are transcribed by RNA polymerase II or III into primary miRNA transcripts (pri-miRNA) that contain hairpin sequences. Pri-miRNAs are cleaved by the RNase (RNase) III Drosha-DGCR8 (DiGeorge syndrome critical region 8) complex into precursor miRNAs (pre-miRNAs) (7, 8).

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Figure 1. A schematic diagram showing the microRNAs that specifically affect pulmonary artery smooth muscle cells (PASMCs), endothelial cells (ECs), and fibroblasts and contribute to the pathogenesis of pulmonary arterial hypertension (PAH). Red arrows indicate up-regulation; green arrows indicate down-regulation.

Pre-miRNAs are transported to the cytoplasm by exportin 5–Ran-GTP and processed to double-stranded mature miRNAs by the RNase Dicer in complex with the double-stranded RNA-binding protein TRBP (7, 8). After the separation of the two strands by helicases, the passenger strand is degraded, whereas the functional (guide) strand of the mature miRNA is loaded with Argonaute (Ago) 2 proteins and incorporated into the RNA-induced silencing complex. In the RNA-induced silencing complex, through complementary sequences, the guide strand of miRNAs recognizes and binds to the 3′-untranslated regions of their mRNA targets, leading to mRNA destabilization, partial mRNA degradation, and repression of protein translation (7, 8). In recent years, a noncanonical pathway of miRNA biogenesis has been reported (9, 10). Short introns with hairpin are spliced and debranched to yield mirtron hairpins, which mimic the structural features of pre-miRNAs. This process appears to be independent of Drosha-mediated cleavage. Mirtron is exported to cytoplasm by exportin-5 and processed to mature and functional miRNAs (9, 10). Although mirtrons were originally identified in flies and nematodes, a recent report suggested that there are abundant mirtrons in the

human and mouse (11). The miRNA pathway is evolutionarily conserved and regulates many aspects of cellular function, including cell cycle progression and cellular differentiation, proliferation, survival, and metabolism (12, 13). During evolution, the sequence and expression pattern of many miRNAs are conserved throughout phylogeny, and miRNA diversity correlates with speciation. Increases in miRNA numbers are generally associated with increased structural complexity over evolution time (14). To date, miRBase has reported ~2,000 human miRNAs in its release version 20 (15). It is estimated that over 30% of human genes are regulated by miRNAs (5). The expression patterns of miRNAs are regulated by many factors, such as the developmental stage and age, gender, and environmental factors, and display spatiotemporal features. For example, expression levels of microRNA-17–92 (miR-17–92) are high in embryonic stem cells and midgestation embryos and relatively low in mature tissues and during aging (16, 17). The miR-17–92 cluster is also highly expressed during T cell activation and silenced during memory development (18). In the lung, a group of miRNAs are differentially expressed between males and females, presumably by retinoic,

IGF1R, Tp53, and Akt pathways (19). Hypoxia is known to alter the expression of a number of miRNAs (20). Therefore, miRNAs may be important effectors in the regulation of gene expression with respect to development and aging, sex differences, response to exogenous environmental influences, and human diseases.

Within an organism, there are multiple organs/tissues/cells that exhibit organ-, tissue-, and cell-specific gene expression signatures. Because miRNAs regulate multiple mRNA targets, it is reasonable to speculate that expression of miRNAs is also organ-, tissue-, and cell specific. Landgraf and colleagues reported the sequences of over 250 small RNA libraries from 26 different organ systems and cell types and reported a cell- and tissue-specific miRNA expression pattern in mammals. They found that more than 97% of all miRNA originated from fewer than 300 miRNAs, suggesting a ubiquitous expression of abundant miRNAs, such as miR-16 and miR-21 (21). Although very few miRNAs were exclusively expressed in given tissues and cells, a third of miRNAs demonstrated a certain degree of tissue specificity (e.g., miR-224 in the respiratory system and miR-142, miR-144, miR-150, miR-155, and miR-223 in hematopoietic cells) (20). More importantly, the expression levels of miRNAs inversely correlate with their predicted mRNA targets in a given tissue, as reported by Sood and colleagues (22). There are exceptions: expression of miR-124, miR-133, and miR-206 parallels the expression of their predicted targets in the developing central nervous system, and a few targets are expressed at even higher levels in the presence of their upstream miRNAs (23). This miRNA-mRNA coexpression phenomenon may suggest a possible negative feedback response, indicating a complex regulatory circuit between miRNA and mRNA (23, 24). Under pathological conditions such as cancer, miRNA expression is dysregulated. miR-17–5p, miR-19a/b, miR-18a/b, and miR-25 are generally up-regulated in cancer, whereas other miRNAs display a cancer type-specific pattern (e.g., miR-181a-1 [4] was absent from B-cell chronic lymphocytic leukemia, miR-126 was highly expressed in precursor B-cell acute lymphoblastic leukemia, levels of miR-150 were reduced

Figure 2. The biogenesis of microRNAs (miRNAs). In the nucleus, miRNA genes are transcribed into primary miRNA transcripts (pri-miRNA) by RNA polymerase II or III. Pri-miRNAs are processed into precursor miRNAs (pre-miRNAs) by the Drosha-DGCR8 complex. Pre-miRNAs are exported by exportin 5–Ran-GTP to the cytoplasm, where they are processed to double-stranded mature miRNAs by Dicer and TRBP. The passenger strand is degraded, whereas the functional strand of the mature miRNA is loaded with Argonaute (Ago2) proteins and incorporated into the RNA-induced silencing complex (RISC), where the functional strand of miRNAs recognize and bind to the 3'-untranslated regions of their mRNA targets, leading to target mRNA destabilization, mRNA degradation, and repression of protein translation.

in Burkitt's and diffuse large B-cell lymphoma) (21).

Dysregulation of miRNA in Pulmonary Hypertension

There are reports that the expression profile of miRNAs in human lungs with PAH is altered compared with normal controls. Courboulin and colleagues compared 337 miRNAs between PAH and control lungs and identified six miRNAs that were up-regulated and only one miRNA, miR-204, that was down-regulated in PAH (25). Rhodes and

colleagues performed a microarray screen on plasma miRNAs from eight patients with PAH and eight healthy control subjects and identified 58 miRNAs that were dysregulated, with plasma miR-150 levels being reduced most significantly in patients with PAH (26). Other studies have indicated that miR-17-92, miR-143/145, miR-21, and many other miRNAs are dysregulated in PAH (25, 27–34); however, only a handful of these miRNAs has been confirmed to play a role in PAH using a variety of experimental models of PH (Table 1). miR-17-92, miR-143/145, miR-21, and miR-124 have been extensively studied

and hundreds of targets have been identified, whereas miR-204, miR-424, and miR-503 are less studied. We have listed the functional targets of these miRNAs in Table 2.

Caruso and colleagues used models of PH induced by hypoxia and monocrotaline (MCT) in rats and found that chronic hypoxia reduced the expression of Dicer, suggesting an overall down-regulation of miRNAs in PAH (30). Subsequently, miR-204 was shown to be down-regulated in hypoxia- and MCT-induced PH (25). Although some miRNAs are consistently up-regulated (miR-322 and miR-451) or down-regulated (miR-22, miR-30, and let-7f) in hypoxia and MCT-induced PH, there are some significant differences in the miRNA expression profiles between these two models (30).

A more definite causative role for miRNAs in the pathogenesis of PAH is supported by studies where inhibition of miR-21, miR-145, or miR-17, and miR-20a of the miR-17-92 cluster prevents or reverses existing PAH (27, 32, 35–37). Altered expression of miRNAs is also associated with plexiform vasculopathy with severe PAH (38). Expression levels of miR-143/145 and miR-204 are higher in concentric lesions than in plexiform lesions, whereas miR-126 and miR-21 are higher in plexiform lesions than in concentric lesions (38), suggesting a potential role for miRNAs as diagnostic or prognostic markers in PAH.

Careful assessment of the role of miRNAs in the pathogenesis of PAH requires accurate and reliable methods to detect and measure levels of miRNAs. Generally, levels of miRNAs can be measured by microarray analysis, deep-sequencing, real-time PCR, Northern blot analysis, and *in situ* hybridization (39). Microarray analysis can simultaneously measure a large number of miRNAs and is most often used to obtain the global miRNA expression pattern as a prelude to validation by other techniques, such as real-time PCR. Deep sequencing generates massive small RNA sequences from a given sample and measures absolute abundance of given miRNAs and thus has an advantage over microarray analysis to detect miRNAs with low copy numbers. Hence, deep sequencing is a powerful tool to identify novel miRNAs.

Table 1. A List of Dysregulated MicroRNAs in Pulmonary Hypertension

microRNA	Changes in PH	Sample Type	Targets	Function	References
miR-17-92	↓ Transient ↑	Human PAH PSMCs Hypoxic PH mouse lung homogenates	PDLIM5 p21	PASMC differentiation PASMC proliferation	27, 28 26
miR-145	NA ↑	Human PAECs Human HPAH and IPAH lung tissue and PSMCs	BMPR2 KLF4, Smad4, Samd5	PAEC proliferation PASMC differentiation and inhibition of PASMC proliferation	58 31
miR-21	↑ ↑	Hypoxic PH mouse PSMCs BMPR2 mutation PAH PSMCs Hypoxic human PSMCs Human pulmonary vessels (< 200 μm) MCT-PH rat lung homogenates Hypoxia/SU5416 mouse lung homogenates IL-6 transgenic PH mouse lung homogenates	PDCD4, SPRY2, PPARα RhoB	PASMC proliferation Decrease angiogenesis and vasodilatation	42 34
miR-204	NA ↓	PAECs Human PAH PSMCs	PDCD4/caspase-3 SHP2	Antiapoptosis in PAECs Apoptosis and inhibition of PASMC proliferation	59 24
miR-424/miR-503	↓	Human PAH lung biopsies Hypoxic PH mouse lung homogenates MCT-PH rat lung homogenates Human PAH PAECs	FGF2/FGFR1	Antiproliferative and proapoptosis Antiproliferation	62
miR-124	No change ↓	Human PAH PSMCs Human PAH fibroblasts	Monocyte chemotactic protein-1 and polypyrimidine tract-binding protein 1	Antiproliferative	64

Definition of abbreviations: HPAH, heritable pulmonary arterial hypertension; IPAH, idiopathic pulmonary arterial hypertension; MCT, monocrotaline; NA, not available; PAEC, pulmonary artery endothelial cell; PAH, pulmonary arterial hypertension; PASMC, pulmonary artery smooth muscle cell; PH, pulmonary hypertension.

Although Northern blotting is time consuming and suffers from low sensitivity, it is useful to visualize mature miRNAs and pre-miRNAs. Because miRNA expression is tissue- or cell specific and many of them play a tissue specific role in PAH as discussed in this review, measuring miRNA levels in a tissue- or cell-specific manner is useful, especially when combined with *in situ* hybridization. However, given the small size of target miRNA sequences, *in situ* hybridization may have low specificity, and therefore it is technically challenging to gain accurate results. Quantitative real-time PCR is by far the most popular method to measure miRNA levels and can be performed by SYBR green or Taqman probes. Two common methods were developed to synthesize cDNAs from miRNAs: (1) the poly(A) method, in which miRNAs are polyadenylated by poly(A) polymerase, followed by cDNA synthesis with a universal oligo(dT) primer, and (2) the

stem-loop method, in which stem-loop primers are used for cDNA synthesis. The poly(A) method is suitable for high-throughput profiling of miRNA expression, yet it is less specific due to cDNA synthesis of all polyadenylated RNAs, including mRNA; rRNA; tRNA; pri-, pre-, and mature miRNAs; and a universal reverse primer. Stem-loop RT primers are specific for an individual miRNA, but the efficiency of the stem-loop method is relatively low and costly. Kang and colleagues reported a modified quantitative PCR assay for miRNA quantification, namely the S-Poly(T) miRNA assay. In this method, miRNAs are polyadenylated, and cDNAs are synthesized with a S-Poly(T) primer. cDNAs are then amplified by a universal reverse primer, a universal Taqman probe, and a miRNA-specific forward primer containing an oligo(dT)₁₁ sequence and six miRNA-specific bases. This method is reported to have a 4-fold increase in

sensitivity compared with traditional methods (40).

miRNA Regulation of PASMC Phenotype

The miR-17-92 Cluster

The miR-17-92 cluster contains six mature miRNAs (miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92a) that are transcribed as one common primary miRNA until it is processed to mature miRNAs (41). Although miR-17-92 is known to be oncogenic, recent studies suggest that miR-17-92 also plays an important role in lung development and in the pathogenesis of PAH (16, 28, 29, 42). Loss of miR-17-92 leads to severe hypoplastic lungs in mouse embryos, and these mice die shortly after birth (16). miR-17-92 induces proliferation of lung epithelial cells and inhibits differentiation of lung

Table 2. Other Functional Targets of Lesser-Known MicroRNAs That Participate in the Development of Pulmonary Hypertension

MicroRNA	Targets	References
miR-204	RUNX2	6, 88, 89
	SOX4	90, 91
	angiopoietin-1	92
	Cdc42	93
	TrkB	94, 95
	Slug	96
	Mcl-1	97
	MAFA	98
	SIRT1	99
	Sox11	100
	BDNF	101
	SMAD4	102
	Bcl-2	95, 103
	LC3B	104, 105
	AP1S2, Bcl2l2, BIRC2, EDEM1, EZR, FZD1, M6PR, RAB22A, RAB40B, SERP1, TCF12, and TCF4	106
	Meis2	107
	TGF- β R2 and SNAIL2	52
miR/424 and miR-503	BCL-2, IGF1R	108
	Rictor	109
miR-424	cdc25A	110, 111
	PDCD4	112
	c-Myb	113
	VEGF, KDR	114
	FGFR1	115
	CDX2	116
	CX3CL1	117
	cyclin D1	118
	Chk1	119
	MEK1 and cyclin E1	120
	cullin 2	121
	NFI-A	122
miR-503	IGF1R and BCL2	123–125
	Smurf2	126
	PI3K p85 and IKK- β	127
	FANCA	128
	RANK	129
	CCND1	130
	CUGBP1	131
	CCNE1	110

epithelial progenitor cells (16). We have reported that miR-17-92 is down-regulated in PSMCs isolated from patients with PAH (28, 29). We found that reduced expression of miR-17-92 in patients with PAH is associated with decreased levels of α -smooth muscle actin (α -SMA), SM22 α , and calponin, suggesting a correlation between a dedifferentiated smooth muscle cell phenotype and loss of miR-17-92 expression. Conversely, overexpression of miR-17-92 restores the differentiated phenotype. Our results also showed that miR-17-92 binds to the 3'-untranslated regions region of PDZ and LIM domain

protein 5 (PDLIM5) and inhibits its expression. Furthermore, PDLIM5 is essential for miR-17-92-mediated TGF- β /SMAD, signaling and PSMC phenotype (28, 29). Pullamsetti and colleagues reported that miR-17 is transiently up-regulated in the hypoxia-induced PH mouse model. Inhibition of miR-17 prevented and partially reversed hypoxia-induced PH, presumably by inducing p21 to inhibit proliferation of PSMCs (27). Therefore, miR-17-92 appears to contribute to PAH by two mechanisms: (1) in the initial stage of remodeling of blood vessels, up-regulation of miR-17-92 promotes

PASMC proliferation, and (2) in the later progression stage, reduced miR-17-92 increases PDLIM5 to maintain PSMCs in a dedifferentiated state.

MicroRNA-21

In normal human PSMCs, hypoxia induces miR-21 by 3-fold, with a decrease in expression of miR-21 targets such as programmed cell death protein 4 (PDCD4), Sprouty 2 (SPRY2), and peroxisome proliferator-activated receptor- α (PPAR α). Treatment with anti-miR-21 inhibits hypoxia-induced PASMC proliferation and migration and increases the expression of its target proteins (43). Inhibition of miR-21 prevents and reverses hypoxia-induced PAH (37). Thus, elevated levels of miR-21 may contribute to PASMC proliferation and migration and to the development of PAH (43).

MicroRNA-124

The nuclear factor of activated T cells (NFAT) signaling pathway has been implicated in PASMC proliferation and PAH. Kang and colleagues adopted a systematic approach to identify miRNAs that regulate the NFAT pathway using a luciferase reporter. Among eight unique miRNAs that modulate NFAT activity, miR-124 exhibits a robust inhibition of NFAT activity, dephosphorylation, nuclear translocation, and inhibition of NFAT-dependent transcription of IL-2. miR-124 appears to directly target NFATc1, CAMTA1 (calmodulin-binding transcription activator 1), and PTBP1 (polypyrimidine tract-binding protein 1) to modulate the NFAT pathway. During hypoxia, miR-124 is down-regulated in human PSMCs and mouse lungs. Overexpression of miR-124 inhibits human PASMC proliferation. The antiproliferative effects of miR-124 may be useful in developing treatments for PAH (44).

The miR-143/145 Cluster

miR-143 and miR-145 are organized in a polycistronic cluster and are controlled by a common promoter region and transcribed as one common primary miRNA (45). The miR-143/145 cluster locates within a 1.7-kb highly conserved region of mouse chromosome 18 (46) or human chromosome 5q33 (47). Because miR-143/145 is most abundantly expressed in heart,

vascular, and visceral SMCs, it has been labeled as a SMC-specific miRNA (48, 49). A few downstream targets of miR-143 and miR-145 have been identified, and miR-143/145 has been shown to be necessary for maintenance of the contractile phenotype of SMCs and for its fate (45, 50). For example, in rat aorta vascular SMCs, miR-145 inhibits KLF5, resulting in induction of myocardin and increased expression of SMC markers such as α -SMA, calponin, and smooth muscle myosin heavy chain (49). Overexpression of miR-145 prevents cell growth *in vitro* (49). Another study suggests that miR-143 (by inhibiting Elk-1) and miR-145 (by inhibiting KLF4 and CamKII- δ) inhibit SMC proliferation but promote differentiation (45). miR-143/145 expression is up-regulated in human PAH and hypoxia-induced mouse PH (25, 32). Patients with PAH who have bone morphogenetic protein receptor type II (BMPR2) mutations have increased miR-145 levels. Down-regulation of BMPR2 also induces miR-145 in human and mouse PSMCs, suggesting that miR-145 is a downstream target of BMP signaling. Indeed, Davis-Dusenbery and colleagues discovered that, in vascular smooth muscle cells, TGF- β and BMP4 induce myocardin (Myocd) expression or nuclear translocation of Myocd-related transcription factors (MRTFs), respectively, resulting in increased transcription of pri-miR-143/145 and higher levels of mature miR-143 or miR-145, repression of KLF4 expression, and increased contractile gene expression (51). In addition, although manipulation of miR-145 does not appear to alter baseline pulmonary vascular function, anti-miR-145 but not anti-miR-143 inhibits hypoxia-induced PH in mice (32).

miR-204

miR-204 locates within the intronic region of TRPM3 (transient receptor potential melastatin 3), shares the same regulatory mechanism as TRPM3, and is down-regulated by STAT3 (25, 52). miR-204 is primarily expressed in PSMCs, and its expression levels are reduced in human PAH and in hypoxia- and MCT-induced PH in rats. Prohypertensive signaling molecules, such as PDGF, endothelin-1 and angiotension II, decrease miR-204 expression, and reduction of miR-204 activates Src-STAT3-NFAT pathway via up-regulation of SHP2

(Src activators), leading to PSMC proliferation and increased resistance to apoptosis. miR-204 mimics can reverse MCT-induced PH (25). miR-204 levels appear to be negatively correlated with the severity of human PAH and the amount of miR-204 in buffy coat cells mirrors the levels in PSMCs. Lee and colleagues reported that mesenchymal stromal cell-derived exosomes, but not fibroblast-derived exosomes, increase lung levels of miR-204, suppress STAT3 signaling induced by hypoxia, and inhibit vascular remodeling and hypoxic pulmonary hypertension (PH) (53). These studies suggest that miR-204 might be used as a diagnostic marker for PAH and that it may be a potential candidate in the treatment of this disease (25, 53).

miR-210

miR-210 is consistently and reproducibly induced by hypoxia in various cell types (54). miR-210 is robustly induced by hypoxia in PSMCs and mouse lungs via an HIF-1-dependent pathway. Up-regulation of miR-210 suppresses E2F3 and increases resistance to apoptosis, resulting in hyperplasia of PSMCs (55).

miRNA Regulation of Pulmonary Artery Endothelial Homeostasis in PAH

The miR-17-92 Cluster

miR-17, miR-18, miR-19, and miR-20 expression is increased upon the induction of endothelial cell differentiation of murine embryonic stem cells or induction of pluripotent stem cells. In contrast, miR-92a and the primary miR-17-92 transcript were down-regulated, suggesting that miR-17-92 may be involved in angiogenesis (56). In endothelial cells, VEGF induces miR-17-92 expression, and miR-17-92 is required for endothelial cell proliferation and angiogenesis (57). A fraction of patients with PAH have mutations of BMPR2, and down-regulation of BMPR2 is associated with PAH (58). Brock and colleagues have discovered that miR-17-5p and miR-20a, two members of the miR-17-92 cluster, directly target BMPR2 in PAECs. They showed that IL-6, a prohypertensive cytokine, up-regulates miR-17/92 expression via STAT3 (signal transducer and activator of

transcription 3). The promoter region of the miR-17/92 gene (C13orf25) contains a highly conserved STAT3-binding site, and persistent activation of STAT3 induces miR-17-92 (59). These results suggest that inhibition of BMPR2 results in up-regulation of miR-17-92, which increases PAEC proliferation and makes them resistant to apoptosis, resulting in PH (Figure 3).

miR-21

Although multiple factors contribute to the pathogenesis in PAH, it is not clear how these multiple pathways are interlinked. Parikh and colleagues adopted a network bioinformatics approach to identify miR-21 as a key miRNA controlling multiple functional pathways associated with hypoxia, inflammation, and genetic haplo-insufficiency of BMPR2. First, they compiled a list of 131 genes that are implicated in the development of PH and constructed a consolidated interactome and mapped the interactions among these genes. Then they used the TargetScan 5 algorithm to predict the miRNAs that regulate these genes. Out of a select group of 29 miRNAs predicted to control expression of a convergent set of pathways in PH, they chose to study miR-21 and found that miR-21 is up-regulated in pulmonary tissue from several rodent models of PH and patients with PAH. Hypoxia and BMPR2 signaling up-regulate miR-21 in cultured PAECs, whereas miR-21 down-regulates BMPR2 expression, forming a negative feedback loop. miR-21 directly targets and suppresses RhoB expression and Rho-kinase activity, resulting in decreased angiogenesis and vasodilatation. Loss of miR-21 increases RhoB expression and Rho-kinase activity, exaggerating hypoxia/SU5416-induced PH (35). A recent study reported that deletion of miR-21 in mice results in activation of the PDCD4/caspase-3 axis in PAECs and induces progressive PH. Conversely, overexpression of miR-21 reduces PDCD4 expression and protects mice from PH in the hypoxia/SU5416 model (60). Thus, miR-21 appears to act as a brake to inhibit the progression of PH. However, other studies suggest that miR-21 is down-regulated in MCT-induced rat PH and in endothelial cells of human PAH (30, 31) and that inhibition of miR-21 prevents and reverses hypoxia-induced PH (37). Sarkar and colleagues showed a 3-fold up-regulation of miR-21 in hypoxic human PSMCs; however, Caruso and colleagues

Figure 3. Molecular mechanisms involved in the role of miR-17-92 in PAH. miR-17-92 is transiently up-regulated in hypoxia-induced pulmonary hypertension, presumably by IL-6, c-Myc, or E2F1. Up-regulation of miR-17-92 inhibits BMPR2 and increases pulmonary artery EC proliferation and their resistance to apoptosis. In PASMCs, the role of miR-17-92 is 2-fold: miR-17-92 inhibits the expression p21 to promote PASMC proliferation. miR-17-92 also inhibits PDLIM5 to induce TGF- β /SMAD signaling, inducing the expression of smooth muscle cell contractile proteins.

did not find any change in miR-21 expression in hypoxic rat lungs or rat PASMCs, suggesting a differential regulation of miR-21 between two species (30, 43). Caruso and colleagues also reported that miR-21 is down-regulated in rat lungs of MCT-induced PH but not in hypoxia-induced PH, whereas Parikh and colleagues showed that miR-21 is induced in MCT-treated rats in a time-dependent manner (30, 35). It is unknown whether this discrepancy is due to the different dose of MCT (60 mg/kg in the latter study; unspecified in the former study) or the different normalization methods during miR-21 measurement (miR-21 expression is normalized to U87 in the former study and Rnu48 in the latter study). The anti-PH role of miR-21 is demonstrated in the hypoxia/SU5416 model (35, 60), whereas the pro-PH role of miR-21 is shown in hypoxia alone model (37). This contradiction may be partially explained by the difference in PH severity and histopathological changes between hypoxia/SU5416- and hypoxia-induced PH. The

hypoxia/SU5416 PH model is characterized by exacerbated PAEC proliferation, which more closely mimics human PAH. In PAECs, miR-21 directly suppresses RhoB expression and Rho-kinase activity and the PDCD4/caspase-3 axis, resulting in inhibition of PH (35, 60). However, in the hypoxic PH model, the PAEC proliferation is minimal and PASMC proliferation is relatively dominant. Therefore, inhibition of miR-21 prevents PASMC proliferation and migration, resulting in inhibition of PH (36). These seemingly contradicting conclusions may result from the different roles of miR-21 in the various cell types that participate in the pathogenesis of PAH. Thus, the role of miR-21 in PAH is complex, and further studies are warranted to fully elucidate the involvement of miR-21 in PAH in a spatial and temporal manner.

miR-27a

In PAECs, hypoxia induces miR-27a to increase cell proliferation and expression of endothelin 1 and to decrease PPAR γ levels (61). Inhibition of miR-27a prevents

hypoxia-induced PAEC proliferation and endothelin 1 expression. More importantly, rosiglitazone, a PPAR γ ligand, attenuates hypoxia-induced miR-27a, whereas miR-27a levels are up-regulated in PPAR γ knockout mice. These results suggest that a negative feedback loop between miR-27a and PPAR γ may provide an amplifying signal to promote PH (61). However, another study showed that in PAECs and PASMCs from patients with HPAH, knockdown of BMPR2, a mutation responsible for HPAH decreased BMP2-induced miR-27a expression and that miR-27a exhibits antiproliferative effects (31). In their model, induction of *miR-27a* appears to signal growth suppression that is lost in HPAH (31).

miR-424 and miR-503

The apelin (APLN)-apelin receptor (APLNR) signaling axis is known to maintain pulmonary vascular homeostasis (62). In patients with PAH, reduction of APLN causes hyperproliferative and antiapoptotic phenotype of PAECs. APLN knockout exacerbates hypoxia-induced PH (62). Further studies suggest that persistent low levels of APLN-APLNR axis lead to reduced levels of miR-424 and miR-503, which directly suppress FGF2 and FGFR1. In PAH, down-regulation of miR-424 and miR-503 induces FGF2 and FGFR1 and subsequent proliferation of PAECs and PASMCs. Accordingly, restoration of miR-424 and miR-503 attenuates MCT- and hypoxia/Sugen 5416-induced PH (63). FGF2 is overexpressed in PAECs of patients with IPAH, and elevated levels of FGF2 contribute to PAH by both autocrine and paracrine mechanisms: in IPAH PAECs, elevated levels of FGF2 induce PAEC proliferation and resistance to apoptosis, whereas suppression of FGF2 decreases PAEC proliferation and resistance to apoptosis. In PASMCs, elevated secretion of FGF2 induces PASMC proliferation (64). Together, these results suggest a critical role for the APLN-miR-424/503-FGF2 pathway in the pathogenesis of PAH and miR-424 and miR-503 are negative regulators of PAH.

miRNAs That Regulate Fibroblasts in PAH

miR-124

In PAH, remodeling of adventitia is associated with activation of adventitial

fibroblasts, leading to increased cell proliferation, migration, and secretion of inflammatory cytokines. A recent report indicated that levels of miR-124 are reduced in fibroblasts isolated from patients with PAH and experimental PH models (65). Reduced miR-124 causes hyperproliferation and migration of fibroblasts, whereas overexpression of miR-124 inhibits proliferation and migration of fibroblasts (65). miR-124 can directly suppress expression of monocyte chemotactic protein-1 and polypyrimidine tract-binding protein 1, which regulate Notch1/phosphatase, tensin homolog/FOXO3/p21Cip1, p27Kip1 signaling, and fibroblast proliferation and migration (65). In addition, miR-124 appears to be suppressed by histone deacetylases, and treatment of histone deacetylase inhibitors restores miR-124 expression and suppresses fibroblast proliferation, suggesting a potential therapeutic role for histone deacetylase inhibitors in PAH (65).

Although in this review we focus on the contributions of PSMCs, PAECs, and fibroblasts in the pathogenesis of PAH, other reports provide compelling evidence that inflammatory cells are also critical in PAH (66, 67). Pericytes are mainly located on the external surface of small blood vessels with elongated and multibranched morphology and have been known to regulate vascular development and remodeling (68). In a subset of patients with Adams-Oliver syndrome at high risk for PH, abnormal pericyte recruitment contributes to pathogenesis of PH (69). Recently, Ricard and colleagues demonstrated that pericyte coverage is increased in PH and serves as a source of smooth muscle-like cells (70). More importantly, different cell types may communicate with each other in the pathogenesis of PAH. Hergenreider and colleagues reported that in endothelial cells Krüppel-like factor 2 (KLF2) binds to the promoter of the miR-143/145 cluster and up-regulates the expression levels of miR-143/145. KLF2-transduced or shear stress-stimulated human umbilical vein endothelial cells secrete extracellular vesicles enriched with miR-143/145, which control SMC marker expression in cocultured SMCs (71). This important finding suggests a complexity in miRNA regulation and points to a potential strategy of using a miRNA- or

extracellular-vesicle-mediated mechanism to treat PAH.

Future Directions

Regulation of miRNAs in PAH

Although evidence of participation of miRNAs in PAH is emerging, the regulation of miRNAs in PAH is less known. Brock and colleagues found that IL-6, a cytokine involved in the pathogenesis of PH, induces miR-17-92 via STAT3 (signal transducer and activator of transcription 3)-mediated transcription and represses protein expression of BMPR2. This study provides a mechanistic explanation for the loss of BMPR2 in the development of PH (59). Recent studies suggest that miR-17-92 can be regulated by c-Myc and E2F1 (72, 73); however, the implication of this in regulation of PAH remains unknown. miR-143 or miR-145 are induced by TGF- β /Myocd and BMP4/Myocd-related transcription factors pathways (51). Davis and colleagues reported a unique mechanism by which TGF- β and BMP signaling induces miR-21. They found that TGF- β and BMP signaling recruits SMAD to pri-miR-21 in a complex with the RNA helicase p68 (also known as DDX5), a component of the DROSHA microprocessor complex, promoting the processing of pri-miR-21 into pre-miR-21 (74). However, the implication of this mechanism in PAH is unclear, and we need to continue to study the molecular and pathobiological significance of altered miRNA expression in PAH. Given that multiple miRNAs are down- or up-regulated during PAH, it is also important to investigate the crosstalk among these miRNAs.

Identification of miRNA Targets

Several bioinformatics tools have been developed to predict the potential targets of individual miRNAs (75), and many targets have been confirmed, indicating the usefulness of these tools. However, there is increasing evidence that these tools are far from perfect in accurately predicting the targets, indicating an urgent need to find better ways to identify and confirm miRNA targets. Kang and colleagues reported a novel approach to identifying new targets of miR-21. They overexpressed biotinylated miR-21 mimic

in PSMCs, recovered miR-21-bound mRNAs by the streptavidin-coated magnetic beads pulldown assay, and identified miR-21 targets by Affymetrix microarray analysis. Using this approach, they not only revalidated the previously validated miR-12 targets such as PDGFR4, TGFBR2, PTEN, BMPR2, etc., but also identified nearly all members of the dedicator of cytokinesis (DOCK) 180-related protein superfamily as targets of miR-21. DOCK family genes are not predicted as targets of miR-21 by conventional target prediction algorithms; instead, miR-21 regulates DOCK genes by base pairing at sites other than the seed sequence. Thus, this approach may serve as a powerful tool to identify canonical and noncanonical miRNA targets (76). Because many cell types function differently, it is conceivable that miRNA targets may be cell specific or context specific, which may explain recent controversies in the role of miR-17-92 in angiogenesis (57, 77). Thus, efforts need to be made to identify and confirm targets in a context-specific manner, which may help us to understand biological functions of miRNAs.

Circulating miRNAs as PAH Biomarkers

Recent reports suggest that extracellular miRNAs associate with protein complexes and are protected from RNase digestion. Therefore, they are stable and abundant in the circulation and can be readily detected. These observations raise the possibility that dysregulated miRNAs may serve as biomarkers for PAH diagnosis or early detection. Indeed, Rhodes and colleagues reported in a pilot study that circulating miR-150 is reduced in peripheral blood of patients with PAH and that reduced levels of miR-150 correlate with poor survival in patients with PAH (26). Schlosser and colleagues reported that circulating miR-26a is reduced in rats with MCT-induced PH and in humans with IPAH and that the levels of miR-26a positively correlate with the 6-minute walk distance (78). More importantly, the expression levels of miR-26a were specifically reduced in lungs of MCT-induced PH rats but not in the hearts and lower levels of miR-26a correlated with increased right ventricular systolic pressure and right ventricular hypertrophy (78), indicating that the

change in levels of circulating miRNAs specifically reflects changes in diseased organs or tissues. This fact will likely increase the use of miRNAs as PAH biomarkers.

miRNA Mimics/Antagonists as Therapeutic Agents

Given the success of miRNA mimics and antagonists in the prevention and inhibition of experimental PH, it is reasonable to speculate that miRNA mimics and antagonists may be novel therapeutic agents for the treatment of PAH in humans. An inherent advantage of using miRNA target drugs is the ability of miRNAs to target multiple genes within a network, making them more efficient. Many strategies have been developed for miRNA-based therapeutics, including antisense oligonucleotides, locked nucleic acid (LNA) anti-miR, miRNA sponges, antagomirs and miR mimics, miRNA expression vectors, and miR-Mask (8, 79). The miR-122 inhibitor miravirsin (Santaris Pharma a/s, Copenhagen, Denmark) based on LNA Drug Platform was the first miRNA-targeted drug to be used in a human clinical trial (80). A recent phase II study was done evaluating the safety and efficacy of miravirsin in 36 patients with chronic Hepatitis C virus (HCV) genotype 1 infection. Miravirsin reduced HCV RNA levels in a dose-dependent manner, and the effect lasted even beyond the duration of active therapy without evidence of viral resistance (ClinicalTrials.gov number, NCT01200420) (81). Currently there are seven clinical trials that are listed as active or completed in ClinicalTrials.gov (search on July 30, 2014), evaluating miR-122 and the clinical course of HCV, safety, dosing, effects on IFN- α null responders, and drug interactions with Telaprevir. A search with “microRNA and lung” retrieved 23 studies, and three of them are in PAH: Study NCT00806312 evaluated the miRNA profile and markers of

inflammation in patients with PAH; study NCT02102672 evaluated right ventricular function, ventricular remodeling, and miRNA profiling in PAH after administration of the fatty acid oxidation inhibitor trimetazidine; and NCT01839110 investigated whether ranolazine can improve the outcome of patients with stable PH, on some specific therapies but with right ventricular dysfunction (RVEF $\leq$ 40%), accompanied by a baseline comparison of the metabolic profiling/miRNA/iPS cells of subjects with and without right ventricular dysfunction.

miRNAs are an attractive new class of drug that have a high therapeutic potential because of their small size, known and conserved sequence, and some preclinical evidence of their role in human diseases. However, synthetic miRNA mimics or antagonists suffer from similar drawbacks as RNAi drugs, such as off-target effects, inefficient drug delivery, and low drug efficacy. Off-target effects of miRNAs mainly arise due to (1) the lack of target specificity due to the short seed sequence, (2) the existence of multiple (even hundreds) targets for a single miRNA, and (3) toxicity from miRNA-induced immune responses. Most miRNA agents are designed to be complementary to their targets and are generally indistinguishable from miRNAs within the same family or those with identical seed sequences. miR-17-92 cluster is known to be up-regulated in many cancers, suggesting a potential cancer therapeutic target; however, miR-17-92 is required for lung development and homeostasis and global knockout of miR-17-92 results in severe hypoplastic lungs in embryos, and these mice die shortly after birth (15), limiting the applicability of miR-17-92 as a drug target. miRNA agents can be detected by both the innate (nucleotide sequence) and adaptive (carrier and/or nucleotide) mammalian immune systems, causing

off-target toxicity (82). Another off-target effect may be caused by the overexpression of miRNA drugs, which may saturate the miRNA biogenesis machinery, altering the expression of other nontargeted miRNAs (82, 83). Many approaches have been developed to limit these off-target effects. To overcome the limitations of targeting mature miRNAs, one strategy may be to target pri-miRNAs and pre-miRNAs. Morpholinos has been shown to inhibit miR-375 by blocking the processing of its pri-miRNAs or the pre-miRNAs, causing defective morphology of pancreatic islet cells. This phenotype has been observed with multiple precursor-targeting morpholinos (84). Targeted delivery of miRNA drugs may also limit unwanted effects. 3'-Conjugation with cholesterol increased the inhibitory efficiency of the miR-122 antagomir in several tissues, particularly in liver (85). Nanoparticle and antibody-based methods are attractive cell- or tissue-specific miRNA drug delivery systems (82). Hornung and colleagues found that immune activation is mediated by the nine nucleotides in the 3' end of the sense strand of siRNA (equivalent to the anti-miR antisense strand) (86). LNA modification of the 2' position of the sugar ring largely reduces the immunostimulatory effects of siRNAs (87). These data provide valuable insight for the design of anti-miR oligonucleotides with minimized immunostimulatory effects. Despite progress in miRNA drug discovery, finding clinically effective miRNA drugs remains a challenge, and further work is warranted. ■

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