

Discerning functional hierarchies of microRNAs in pulmonary hypertension

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Pulmonary hypertension (PH) is a multifaceted vascular disease where development and severity are determined by both genetic and environmental factors. Over the past decade, there has been an acceleration of the discovery of molecular effectors that mediate PH pathogenesis, including large numbers of microRNA molecules that are expressed in pulmonary vascular cell types and exert system-wide regulatory functions in all aspects of vascular health and disease. Due to the inherent pleiotropy, overlap, and redundancy of these molecules, it has been challenging to define their integrated effects on overall disease manifestation. In this review, we summarize our current understanding of the roles of microRNAs in PH with an emphasis on potential methods to discern the hierarchical motifs governing their multifunctional and interconnected activities. Deciphering this higher order of regulatory structure will be crucial for overcoming the challenges of developing these molecules as biomarkers or therapeutic targets, in isolation or combination.

Introduction

Pulmonary hypertension (PH) is a complex and enigmatic vascular disease characterized by pulmonary vascular remodeling and dysfunction, right ventricular hypertrophy and failure, and often death. Exogenous injury (i.e., hypoxia, inflammation, infection) and various other illnesses are linked to PH development, and gene mutations (*BMPR2*, *SMAD9*, etc.) predispose to hereditary forms of PH (1). WHO categorizes PH into five main groups (2) based on these observations, but variation in disease manifestation suggests that multiple factors dictate individualized disease phenotypes. Current medications used for PH are limited to three classes of drugs that all affect pulmonary vasodilation (relaxation) and for the most part are only approved for use in a particularly severe form of PH, pulmonary arterial hypertension (PAH; WHO Group 1), as well as chronic thromboembolic PH (CTEPH; WHO Group 4) (3). These medications do not regress, prevent, or cure the disease.

Attempts to identify the underlying pathobiology of this disease, as well as effective targets for the next generation of PH drugs, has led to the study of an evolutionarily ancient, but only recently appreciated, class of molecules called noncoding RNAs (ncRNAs), of which the most widely studied class includes microRNAs (miRNAs). Embedded throughout the human genome, ncRNAs and miRNAs carry pervasive transcriptional and posttranscriptional regulatory actions relevant to human health and disease, including PH. As our appreciation of the actions of these molecules has advanced, our conception of the complexity of PH has also grown, often causing more confusion, particularly about the varied actions of miRNAs. At the same time, there has been growing evidence of overarching programs of regulation of miRNAs that may allow for mapping of an interconnected hierarchy of miRNA functions. In this review, we define the current state of knowledge of these ncRNAs, with an emphasis on miRNAs, in the pulmonary vasculature. We examine our current understanding of the paradigms of hierarchy, overlap, and redundancy of miRNAs in regulating PH development and progression. Understanding these regulatory motifs will not only facilitate new discoveries in this increasingly complicated disease, but also will be useful for overcoming the challenges of developing these molecules for next-generation clinical diagnostics and therapeutics.

Functional classification of regulatory ncRNAs

Tens of thousands of regulatory ncRNAs are predicted to be expressed in human cells. These classes include miRNAs, long noncoding RNAs (lncRNAs), circular RNAs (circRNAs), and piwi-interacting RNAs (piRNAs), among others (4), and their complex biology is rapidly being elucidated. For example,

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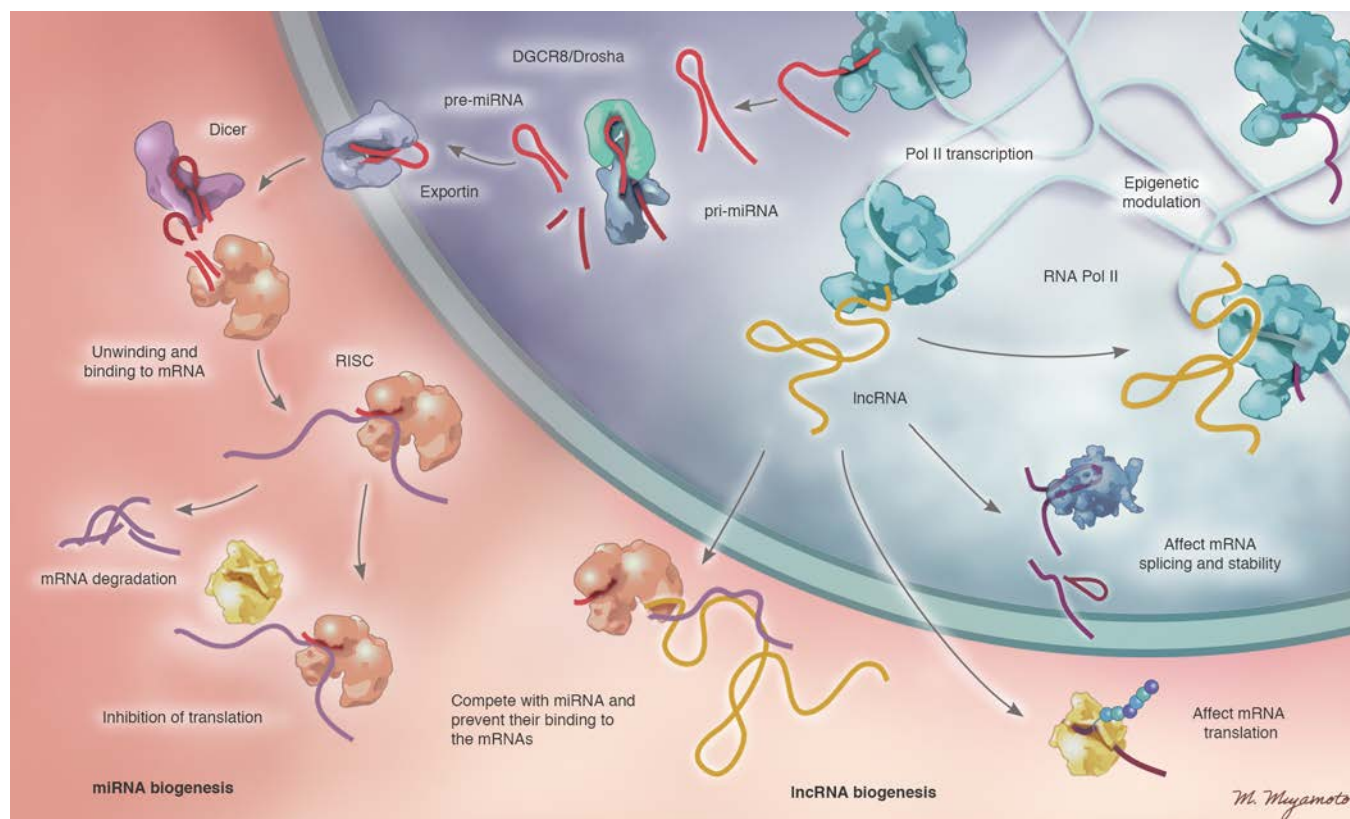


Figure 1. Biogenesis and functions of miRNAs and lncRNAs in PH. (Left) miRNAs are transcribed from the genome in the form of long primary miRNAs (pri-miRNAs), which are then processed by the Drosha/DGCR8 enzyme complex into smaller precursor miRNAs (pre-miRNAs). These small pre-miRNAs are exported into the cytoplasm in an energy-dependent process. In the cytoplasm, pre-miRNAs are acted upon by Dicer to form mature miRNAs. Mature, active miRNAs interact with the RISC (RNA-induced silencing complex), leading to unwinding of duplex miRNAs and binding to the complementary sequence in the 3' untranslated region. Binding of miRNAs to mRNAs can lead to inhibition of translation or mRNA degradation. (Right) Alternatively, after transcription, lncRNAs can interact with DNA or protein molecules directly, often affecting chromatin structure. lncRNAs have also been described to complex with RNAs, such as mRNAs, to influence posttranscriptional splicing and translation or miRNAs to influence target transcript engagement. Illustrated by Mao Miyamoto.

lncRNAs are single-stranded RNAs that are dynamically regulated in the development of PH (5). While some have been shown to have alternative protein-coding functions (6), most lncRNAs affect cellular function by complexing with chromosomal DNA, RNAs, or proteins. lncRNAs often epigenetically regulate gene expression via alteration of chromosomal packaging (7, 8), while others have been described to prevent miRNA binding to target mRNAs (9). Recently, a specific lncRNA, MALAT1, was found to be associated with PH in humans (10) and acts as a competitive inhibitor of miRNA-214, thereby affecting endothelial cell functions (5), including proliferation and migration (11). The biogenesis and emerging functions of lncRNAs are summarized in Figure 1 (12, 13), yet the most advanced insights regarding ncRNAs in the pulmonary vasculature have focused on miRNAs.

miRNAs. miRNAs are small, evolutionarily conserved RNAs that negatively regulate gene expression at the posttranscriptional level. As summarized in Figure 1 (14, 15), miRNAs are transcribed either as a part of an miRNA cluster or alone and can be embedded within introns of protein-coding genes or at entirely separate genomic locations. miRNAs are transcribed as longer primary miRNAs (pri-miRNAs), which are then processed in the nucleus into shorter precursor miRNAs (pre-miRNAs). Pre-miRNAs are then exported into the cytoplasm, where they are cleaved by the protein Dicer into mature miRNAs. Mature duplex miRNAs then interact with the Argonaute (Ago) protein complex, leading to binding of either of the two miRNA strands to specific sequences in target mRNAs, typically in the 3' untranslated regions (3' UTRs). Engagement of miRNAs with target mRNAs can either degrade mRNAs or inhibit their translation. A number of validated online algorithms exist to predict target mRNAs for individual miRNAs (such as refs. 16, 17), and it has been reported that hundreds of target mRNAs may be engaged by a single miRNA (18).

Over the past five years, our appreciation of the pervasive yet complex activity of miRNAs in the pulmonary vasculature has accelerated. We and others have catalogued lists of these miRNAs that have been studied in cultured pulmonary vascular cells, in various animal models of PH, and in human explanted tissues and cells (including refs. 19–22, among others). As these lists have expanded, the interconnected relationships among these miRNAs and their downstream targets and pathways have been challenging to decipher. In this review, we present a conceptual framework for considering groups of these miRNAs and defining potential hierarchical motifs of these molecules.

Cataloguing miRNAs based on their convergent/divergent functions

Convergence on single molecular pathways and cellular phenotypes

As the numbers of miRNAs relevant to PH pathobiology have grown, a predominant hierarchical motif that has emerged includes the functional convergence of discrete cohorts of miRNAs on single molecular pathways relevant to pulmonary vascular pathogenesis. Below are examples of such a convergence on PH-specific pathways and cellular phenotypes. In some cases, a wide range of redundancy appears to be shared among many miRNAs, emphasizing the actions of particular individual miRNAs more as “fine tuners” rather than binary “on/off” biologic switches. In other cases, these analyses have emphasized the context-specific nature of these miRNAs and their targets, suggesting their role in defining the individualized nature of PH manifestations.

BMPR2 signaling. Substantial causative links, both genetic and environmental, exist between PH and dysregulated bone morphogenetic protein receptor 2 (BMPR2) signaling (as reviewed by ref. 23). Loss of function of BMPR2 has been linked to cellular pathophenotypes, including proliferation, cell survival (23), repression of mitochondrial metabolism (24), and endothelial-to-mesenchymal transition (25), among others. However, the comprehensive mechanisms of regulation of BMPR2 that therefore control the specific and often individualized cadence of disease manifestations have yet to be elucidated.

A variety of miRNAs have been causatively linked to BMPR2 expression and signaling in pulmonary vascular cells in vitro and in vivo. Multiple miRNAs directly regulate BMPR2, including the miR-17/92 cluster (26), miR-302-367 cluster (27), and miR-21 (28). In turn, many of these miRNAs were confirmed to modulate PAH in vivo via use of antisense oligonucleotide inhibitor (antagomirs) (29, 30). In addition, miR-21 processing is regulated by BMP signaling globally (31–33) and was shown to control PAH progression in vivo by genetic KO studies (28, 34) and pharmacologic inhibition of miR-21 (35, 36). Despite their modulatory activity in PH, however, actions of these miRNAs do not fully reverse disease manifestations, indicating a level of redundancy and actions as “fine tuners” consistent with their shared targeting of BMPR2. Beyond regulating BMPR2 directly, other miRNAs target other BMP or TGF superfamily signaling components or are themselves modulated by BMP signaling. Under hypoxic conditions, HIF-1 α induces miR-322, which targets BMPR1a and SMAD5, thereby downregulating BMPR2 signaling (37). miR-145 is regulated by BMP4 and is overexpressed in PH, and its genetic deletion prevents the development of PH in mice (38). BMP4 also downregulates miR-96, which in turn inhibits TRB3, a BMP signaling effector, thus affecting a cohort of pulmonary vascular smooth muscle-specific genes (39). Similarly, miR-140-5p targets SMURF1, which regulates BMP signaling and PH in vivo (40). Figure 2A and Table 1 summarize the miRNAs identified to date that are involved in BMPR2 signaling and also carry a causative connection to PAH. It has yet to be determined whether targeted alterations of combinations of these related miRNAs may yield more robust and tailored effects on BMP signaling and PH pathogenesis.

HIF signaling. Chronic hypoxia, or low oxygen exposure, is a key trigger for pulmonary vascular remodeling and PH. It is associated with alteration in various PH-relevant pathways leading to vascular proliferation, metabolic shifts from oxidative phosphorylation to glycolysis, inflammation, and vascular stiffness. The transcription factor HIF-1 α is a major mediator of these effects, and similar to BMPR2 signaling, HIF-1 α also modulates the expression of various miRNAs and reciprocally is regulated by them, as reviewed by ref. 41. For example, miR-424 has been found to stabilize HIF-1 α by targeting its negative regulator and thus promoting angiogenesis in vitro and in vivo (42). In another case, the binding partner of HIF-1 α , HIF-1 β , was reported to be regulated by miR-103/107 (43). Caruso et al. were the first to our knowledge to report substantial numbers of miRNAs dysregulated at various time points in a hypoxic rodent model of PH (44). A number of these miRNAs coincided with prior studies of HIF-dependent miRNAs in cultured pulmonary arterial (45), endothelial (46), and transformed cells (47). While some of these miRNAs, such as miR-210, are direct transcriptional target genes of HIF-1 α , the upregulation of others may be due

Table 1. miRNAs related to BMPR2 signaling

	miRNA	Experimental System	Target(s)	Phenotype	References
1	miR-17~92, miR-20a	HEK293; Rodent model of PH	<i>Bmpr2</i>	miR-17 and miR-20a directly target BMPR2. Therapeutic administration of miR-17 inhibitor improves PH, and miR-20a inhibitor prevents development of PH in vivo.	(26, 29, 123)
2	miR-302	PASMCs	<i>Bmpr2</i>	miR-302 is under the control of BMPR2 signaling and targets BMPR2. It inhibits PASC proliferation and migration.	(27)
3	miR-21	HPAECs; Rodent model of PH	<i>Bmpr2, Rhob</i>	miR-21 is induced by BMP signaling and reduces the expression of BMPR2. miR-21-null mice display greater severity of PH, yet in vivo administration of miR-21 inhibitors attenuates hypoxia induced vascular remodeling.	(28, 34–36)
4	miR-322	PASMCs	<i>Bmpr1a, Smad5</i>	miR-322 promotes proliferation and migration of PASMCs.	(37)
5	miR-145	Human lung samples; Rodent model of PH	multiple	miR-145 expression is increased in PH, and its inhibitor prevents PH.	(38)
6	miR-96	PASMCs	<i>Trb3</i>	BMP4 downregulates miR-96 expression. miR-96 inhibits the contractile phenotype of PASMCs.	(39, 70)
7	miR-130/301	PASMCs from BMPR2 mutant mice; Rodent model of PH PAECs; PASMCs; PAAFs; Rodent model of PH; Human patients samples	<i>Htr1b</i> <i>Pparg, Lrp8</i>	miR-96 mimics ameliorate PH in female mice. BMPR2 controls miR-130/301 expression. miR-130/301 family members regulate vasoconstriction, stiffening, and proliferation in PH. Its inhibitor attenuates PH.	(64–66, 89)
8	miR-140-5p	Human patients plasma sample, Rodent model of PH	<i>Smurf1</i>	miR-140 is reduced in PH; its mimics attenuate PH.	(40)

PAECs, pulmonary artery endothelial cells; PASMCs, pulmonary artery smooth muscle cells; PAAFs, pulmonary artery adventitial fibroblasts.

to the global stabilization of Ago complexes in hypoxia, thus augmenting the processing of miRNAs to their mature active forms (48). Additional complexity likely exists, as more recent studies have suggested. For instance, hypoxia decreases Dicer expression, which in turn reduces the overall biogenesis of miRNAs (49–51). In some cases, this process may be promoted by direct miRNA engagement of Dicer (52).

Among upregulated “hypoxamirs”, substantial efforts have been made to delineate the functions of miR-210 in PH, given its direct and robust link to HIF-1 α and hypoxia. miR-210 was found to have substantial pleiotropic function in mitochondrial metabolism (45), proliferation (53, 54), and cell survival, thus promoting vascular remodeling and PH in vivo (55). miR-210 is also released into the extracellular space and may function as an endocrine or paracrine messenger of hypoxic stress among anatomically distinct tissues (56). Notably, because miR-210 appears primarily active in hypoxia, its pathogenic actions in PH may be limited to subtypes of HIF-relevant PH and may correlate with individualized differences of PH manifestations in WHO Group 3 PH (stemming from hypoxic lung disease) vs. nonhypoxic disease forms. A multitude of additional HIF-dependent miRNAs have been described (Figure 2A and Table 2), but, in general, their relevance in vivo to PH — particularly in humans — has yet to be defined.

Peroxisome proliferator-activated receptor- γ (PPAR γ) signaling. PPAR γ is a nuclear hormone receptor that interacts with effectors, such as the retinoid receptor (RXR), upon binding its ligand, triggering transcriptional activation of its target genes. PPAR γ activates an antiproliferative and proapoptotic program in the pulmonary vasculature, and its reduced expression contributes to PH progression (57, 58). PPAR γ regulates various miRNAs, such as miR-199a (59), miR-98 (60), and miR-21 (61), which, in turn, control effectors of hypoxia, including HIF-1 α , the vasoconstrictor endothelin-1, and the tumor suppressor PTEN, respectively. Reciprocally, PPAR γ is modulated by miRNAs under hypoxic conditions in PH, such as miR-27a (62) and miR-27b (63). Intriguingly, in a computational analysis of miRNAs predicted to target PH-related gene networks, the miR-130/301 family was identified as a master regulator of multiple PH pathways and PH in vivo, by engaging PPAR γ as a central mediator (64–66). Other indirect miRNA-mediated links to PPAR γ signaling have been noted, including downregulation of miR-193-3p in PH after

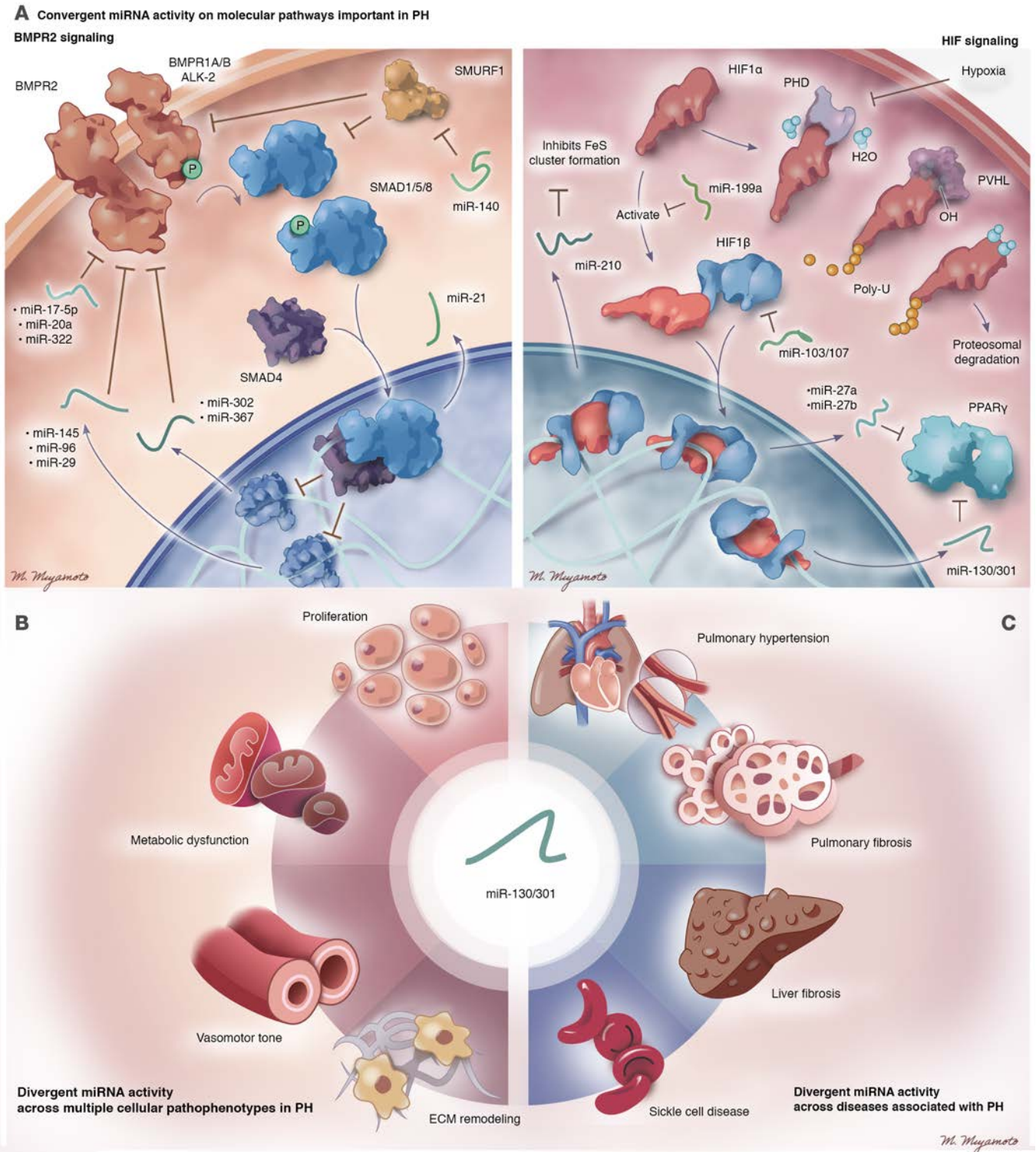


Figure 2. A conceptual schematic to categorize the activities of miRNAs in PH. Currently identified miRNAs that control PH often congregate into higher-order regulatory motifs consistent with convergent or divergent activity across molecular pathways, cellular pathophenotypes, and associated diseases. Representative examples of each category are shown. Such conceptual and often overlapping annotations may be helpful as roadmaps in deciphering the hierarchies of function among sets of miRNAs, their downstream target pathways, and resultant pulmonary vascular phenotypes. **(A)** Convergent miRNA activity on single molecular pathways. Related cohorts of miRNAs exist with convergent activity on BMPR2 signaling (left panel) and HIF signaling (right panel). **(B)** Divergent miRNA activity across multiple cellular pathophenotypes. The miR-130/301 family acts as a system-level regulator of proliferation, vasomotor tone, vascular stiffness, and metabolism across three different pulmonary vascular cell types. **(C)** Divergent miRNA activity across associated disease. The miR-130/301 family controls manifestations of PH, pulmonary fibrosis, liver fibrosis, and sickle cell disease. Illustrated by Mao Miyamoto.

Table 2. miRNAs related to hypoxia

	miRNA	Experimental System	Target(s)	Phenotype	Reference
1	miR-210	Human PAECs; Rodent model of PH; PSMCs	<i>Iscu1/2</i> <i>E2f3, Mkp1</i>	miR-210 represses mitochondrial respiration. Mice deficient in miR-210 are resistant to PH. miR-210 inhibits PASMCM apoptosis.	(45, 55, 53)
2	miR-103/107	PASMCs	<i>Hif1b</i>	miR-103/107 decrease PASMCM proliferation.	(43)
3	miR-21	Human PAECs; Rodent model of PH	<i>Bmpr2, RhoB</i>	miR-21 is increased by hypoxia and targets the HIF pathway.	(28, 35)
4	miR-22	PASMCs; PAAFs	<i>Tgfb1</i>	Hypoxia increases miR-22 expression.	(44)
5	miR-190	PASMCs	<i>Kcnq5</i>	miR-190 is increased in PH and causes vasoconstriction by targeting a voltage dependent K ⁺ channel.	(77)
6	miR-451	Rodent model of PH	Unknown ^A	miR-451 expression is increased in PH. Its inhibitor (but not genetic deletion) attenuates PH.	(44, 124)
7	miR-199a	Human PAECs; PASMCMs; Human patient lung tissue	<i>Smad3</i> <i>Gsk3b^A</i>	miR-199a expression is increased in PH and is inversely correlated with expression of its targets.	(125, 126)
8	miR-98	Human PAECs isolated from human patients; Rodent model of PH	<i>Edn1</i>	PPAR γ decreases miR-98 expression in PH. miR-98 mimics decrease HPAEC proliferation.	(60)
9	miR-130/301	PAECs; PASMCMs; PAAFs; Rodent model of PH; Human patients samples	Pparg pathway	Expression of miR-130/301 is increased in PH and affects vasoconstriction, fibrosis, and proliferation. Its inhibitor attenuates PH.	(64–66, 89)
10	miR-27a	Human PAECs; Rodent model of PH	<i>Pparg</i>	miR-27a expression is increased in PH and is negatively regulated by PPAR γ .	(62)
11	miR-27b	Human PAECs; Rodent model of PH	<i>Pparg</i>	miR-27b expression is increased in MCT model, and its inhibitor reverses PH.	(63)
12	miR-424 and 503	Human PAECs; PASMCMs; Rodent model of PH	<i>Fgf2, Fgfr1</i>	miR-424 and miR-503 is downregulated in PH. They decrease proliferation, and their mimics reduce PH.	(82)
13	miR-138	PASMCs	<i>Mst1</i>	miR-138 decrease PASMCM apoptosis.	(127)
14	miR-124	Human pulmonary artery fibroblast isolated from patients	<i>Ptbp1</i> and <i>Mcp1</i>	miR-124 expression is decreased in fibroblasts from PH patients. Its mimics decrease the proliferation and migration of fibroblasts and HIF-2 α expression.	(74)
15	miR-125a	Human PAECs; PASMCMs; Rodent model of PH	<i>Bmpr2^A</i>	Hypoxia induces miR-125a. miR-125a increases HPAEC proliferation.	(109)
16	miR-206	PASMCs isolated from mouse and human pulmonary arteries	<i>Notch3^A</i>	miR-206 expression is reduced in PH. It decreases proliferation and migration and increases apoptosis of PASMCMs.	(128)
17	miR-223	Human PH lung; Rodent model of PH; PASMCMs	<i>Parp1^A</i>	miR-223 expression is decreased in PH. It reduces the proliferation of PASMCMs, and its mimics ameliorate PH.	(73)
18	miR-204	Human PH lung; Rodent model of PH	<i>Shp2</i>	miR-204 is decreased in PH. Its restoration improves PH.	(129)
19	miR-34a	PASMCs; Rodent model of PH	<i>Pdgfra</i>	miR-34a is downregulated in PH, and it reduces the proliferation of PASMCMs	(130)
20	miR-143	Human PAECs; PASMCMs; Rodent model of PH	multiple	miR-143 is upregulated in PAH. Exosomes containing miR-143 from HPAECs increase the migration of PASMCMs. An miR-143 inhibitor improves PH.	(81)

^ATarget validated in context beyond PH. PAECs, pulmonary artery endothelial cells; PASMCMs, pulmonary artery smooth muscle cells; PAAFs, pulmonary artery adventitial fibroblasts.

administration of apolipoprotein A-I mimic peptides that affected the related transcription factor RXR α (67). Thus, although all of these miRNAs converge upon PPAR γ , how these miRNAs interact with one another and interface with other signaling pathways in PH remains to be determined.

Estrogen signaling. PAH is more prevalent in women as compared with men, but PAH tends to manifest greater severity in men (68, 69). The molecular mechanisms for such sex predisposition are not well defined, but historically, emphasis has focused on the differential effects of sex-specific hormones such as estrogen in the pulmonary vasculature. Recently, miRNAs were found to play a role in estrogen signaling in PH. Estrogen was reported to downregulate miR-96 in pulmonary artery smooth muscle cells (PASMCMs)

isolated from female, but not male, BMPR2 mutant mice. In turn, lower levels of miR-96 directly affected its target serotonin receptor 5-HT1BR, thus promoting PH (70). Another study observed an increase in miR-29 family (miR-29a, -b, -c) expression in female PH patients — an effect amplified by the estrogen metabolite 16 α -hydroxyestrone (16 α OHE) and with downstream effects on the miR-29 target PPAR γ and resultant metabolic vascular phenotypes (71). The role of miRNAs under regulation by other (male or female) sex hormones in PH has yet to be explored in depth. Nonetheless, these initial findings support the notion that miRNA-based tuning of PH differs between males and females and may be essential features of how susceptibility and severity of PAH manifest differently between sexes.

Related cellular phenotypes. Downstream of these molecular pathways, miRNAs also act in concert to affect various cellular pathophenotypes such as mitochondrial and metabolic dysfunction, vascular stiffness, vasomotor tone, proliferation, and apoptosis among others. For example, HIF-related miRNAs — such as miR-210, which affects electron transport (55) — may functionally interface with miR-138 and miR-25, which target the mitochondrial calcium uniporter complex (MCUC), and ultimately promote aerobic glycolysis in PH (72). Alternatively, the HIF-responsive miR-223 was found to induce a separate process of DNA damage in PSMCs, thus linking hypoxia and chromosomal integrity in PH (73).

There is an increasing appreciation that vascular stiffness and extracellular matrix remodeling are early causative events in PH pathogenesis, with both the miR-130/301 family (64) and miR-124 (74) contributing to this phenotype in adventitial fibroblasts. Other miRNAs have been causatively linked to alterations in pulmonary vasomotor tone. For instance, miR-648, miR-199a2, and miR-27a have been linked to the vasoconstrictor endothelin-1 (59, 62, 75), while miR-328 (76) and miR-190 (77) were found to control downstream calcium and potassium influx in contracting PSMCs. Many other miRNAs have been implicated in the control of the vasodilator nitric oxide (78) but with less emphasis thus far on their roles in the pulmonary vascular compartment. Notably, thrombosis and thrombosis-in-situ have been identified as critical PH pathophenotypes, yet rigorous study of miRNAs in platelet biology and the clotting cascade in relation to the pulmonary vasculature has yet to be pursued. Finally, reflecting the advancing molecular parallels between PAH and cancer (79), numerous miRNAs affecting proliferation and cell survival have been implicated in PH, including those targeting chromatin remodeling, such as miR-204 (80), and endothelial to smooth muscle cellular crosstalk, such as miR-143 (81) and miR-424/503 (82). Thus, when overlaying cellular phenotypes onto molecular pathways, this type of miRNA catalog offers a more expansive hierarchical view of their concerted functions and/or exposes deficiencies in our global understanding of this disease.

Divergence of miRNA function controlling multiple molecular pathways

While the convergence of miRNA networks on distinct target pathways has led to a defining of the overlapping functions of these molecules, a parallel investigation of the divergent actions of specific miRNAs in PH has offered separate insights into pathogenesis. Namely, the sheer but specific pleiotropy of miRNAs has driven the notion that there may exist “master miRNA regulators” of PH that drive pathogenesis via control over numerous, seemingly unrelated, molecular pathways. For example, miR-98 and miR-27a/b have been implicated in the control of overlapping pathways involving both hypoxic adaptation and PPAR γ signaling (60, 62, 63). However, utilizing solely traditional reductionist scientific approaches likely limits the search for “master regulators” of PH to only those pathways with already-known molecular links.

For that reason, we have attempted to use both traditional and nontraditional computational approaches in tandem to identify and confirm such miRNA activity in PH. For example, we originally devised a computational method to analyze the architecture of gene networks in order-rank miRNAs with network-wide activity on verified PH-specific genes and their first degree gene interactors (“the PH network, ” mapped using a consolidated set of databases cataloguing all functional interactions reported in the human transcriptome) (28). Our first mathematical iteration identified miR-21 as controlling multiple PH target genes and pathways, including BMPR2 expression and hypoxic reprogramming, which we and others later validated in vitro and in PH rodents in vivo (Tables 1 and 2). Such experimental interrogation also revealed that actions of miR-21 in PH (28, 34–36) and elsewhere (22, 83) are context specific and possibly cell specific. Thus, miR-21, while pleiotropic in its effects in PH, also carries a dynamic and shifting repertoire of actions that makes its functions as a consistent regulator of PH more challenging to define.

Learning from those experiences, we optimized our computational methodology to rank miRNAs based on the number and intercluster spread of their target pools in a larger PH gene network. We identified the miR-130/301 family as the top-ranked miRNAs with actions spanning the entire PH network, and thus highly likely to act as a master regulator of PH (66). We also identified genes in the miR-130/301 target pool in silico by “hubness” and other centrality metrics, thus highlighting the importance of PPAR γ as a direct miR-130/301 target. In turn, this led to the discovery linking this miRNA family to two downstream proliferative pathways in the lung, a molecular axis important in fibrosis and extracellular matrix (ECM) remodeling, and vasomotor tone. Specifically, our analyses indicated that miR-130/301 also serves as a proximal regulator of two subordinate miRNAs important in controlling cellular proliferation in the pulmonary vasculature: miR-424/503 and miR-204. We verified these predictions in vivo and in vitro, demonstrating that the miR-130/301 family was upregulated in the pulmonary vasculature of PH patients (driven by hypoxia, inflammatory cytokines, and BMPR2 deficiency). It modulated vascular proliferation, controlled pulmonary vasomotor tone, and drove a mechanosensitive YAP/TAZ-miR-130/301 axis to promote pulmonary vascular stiffness and a metabolic shift in glutamine consumption (64–66, 84) (Figure 2B). Such system-level discovery in PH provides a glimpse of the extent of miRNA pleiotropy and suggests that the divergent activity of specific miRNAs may provide an opportunity for defining effective diagnostic and therapeutic targets based on their far-reaching influences on multiple PH pathways.

Divergence of PH-relevant miRNA activity among human diseases

Beyond their molecular or cellular landscapes of activity, a cohort of miRNAs is emerging that control shared cellular phenotypes across various diseases. Further identifying miRNAs with shared disease association may offer insights into the relationship of PH to the multitude of its associated secondary diseases or systemic complications beyond the vasculature (2). For example, a number of miRNAs, including let-7 family members (85, 86), are particularly active in both acute pulmonary embolism and CTEPH (WHO Group 4 PH). Additionally, a polymorphism in the 3' UTR in the fibrinogen- α gene (*FGA*) that affects the binding of miR-759 has been associated with CTEPH (87) and pulmonary embolism. In the case of PH associated with sickle cell disease, miR-199a was reported to reduce HIF-1 α expression (59). In perhaps a related context, placental growth factor (PlGF), which is upregulated in sickle cell anemia and may play a key role in promoting PH, has been associated with alterations in miR-648 (75) and the HIF-dependent and PPAR α -dependent miR-301a/454 family (88). Interestingly, in independent work related to chronic lung disease, we demonstrated that the miR-130/301 family can exert pleiotropic control over fibrosis in a network of human diseases, including interstitial lung disease and liver disease (89) — both diseases that carry known clinical associations with PH but with poorly defined molecular underpinnings that link these diseases together. Such insights now highlight the importance of shared miRNA-dependent pathogenic processes across related diseases (Figure 2C).

Beyond the vasculature, alterations of miRNAs have been observed in controlling skeletal muscle abnormalities and right ventricular dysfunction (90) and failure in PH. Via modulation of contractility and angiogenesis, downregulation of miR-208 (91) and miR-126 (92) has been implicated in the transition from a compensated to a decompensated right ventricle (RV). At the same time, downregulation of the endothelial-specific miR-126 was found to impair angiogenic potential in skeletal muscle and was associated with exercise intolerance in PH (93). Further delineation of the shared activity of miRNAs across diseases and in the extra pulmonary compartment may be valuable for establishing molecular links that underlie integral, and potentially surprising, disease associations with PH.

Challenges and opportunities for understanding miRNA biology in PH

The convergent and divergent functions of miRNAs alone offer a wealth of information regarding the pathogenesis of PH, yet because of the sheer number of miRNAs and their target genes, acceleration of these discoveries in the future will necessitate addressing a number of technical and conceptual obstacles (Figure 3).

First, molecular and phenotypic hierarchies could be further unraveled by generation and analyses of data of high-throughput -omics profiling relevant to pulmonary vascular biology. However, attempts at comprehensive molecular profiling in PH have only begun. The deficiency in these data currently has likely led to bias in the choice of which miRNA and target genes are interrogated further — typically focusing on links to already known pathways relevant to PH. Federal initiatives such as the PVDOMICS program (RFA-HL014-030) are a crucial first step to generating these high-density data sets to convince investigators to take a conceptual leap

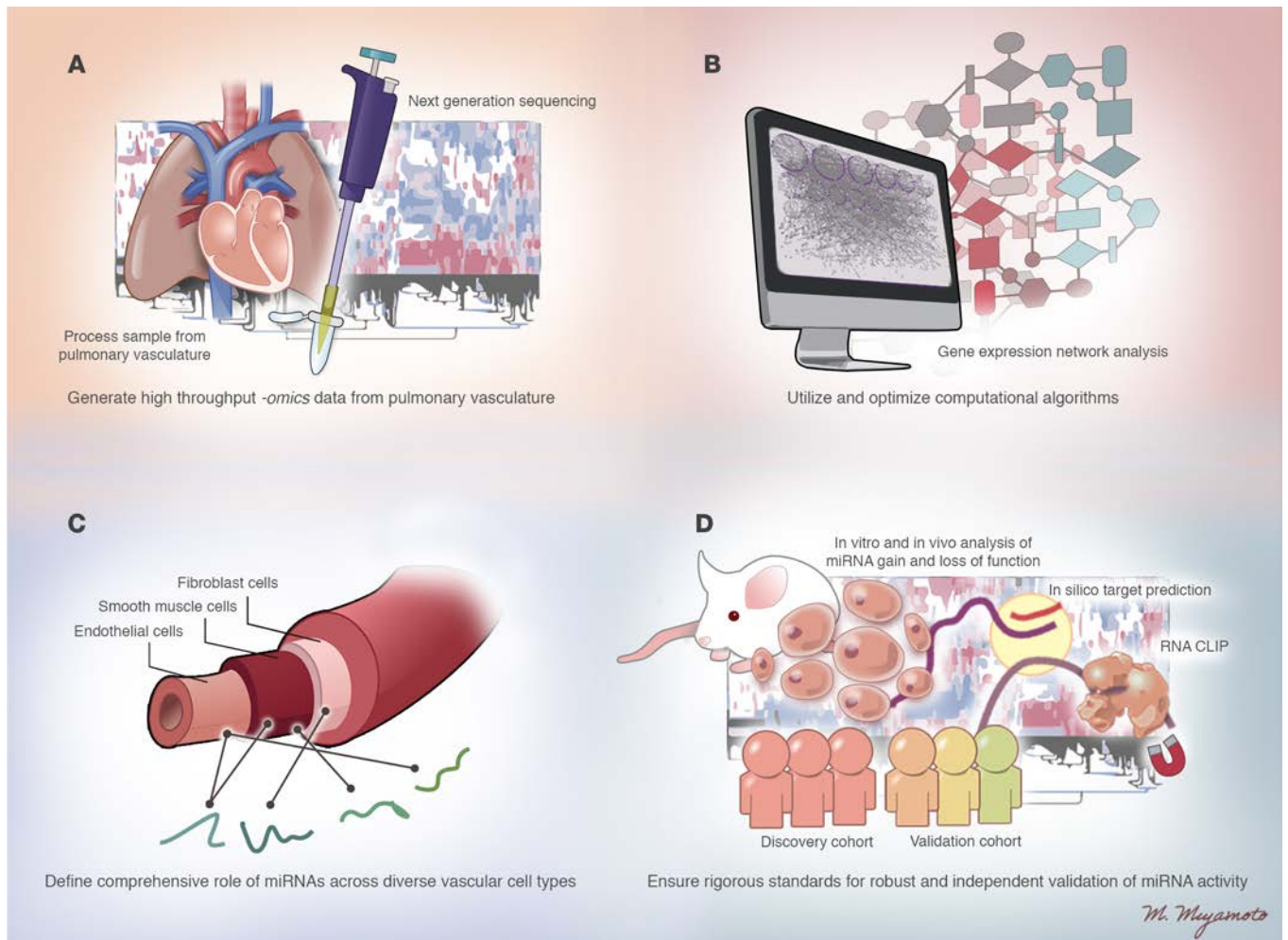


Figure 3. Technical and conceptual challenges for further interrogation of miRNA biology in PH. Current technical and conceptual challenges are listed for addressing the complexity of miRNA activity in PH and ensuring successful development of miRNA-based diagnostics and therapeutics. **(A)** Generation of high throughput -omics data from the pulmonary vasculature, accounting for spatial (cell type specific) and temporal (time dependent) events in PH progression. **(B)** Utilization and optimization of computational algorithms for reliable in silico processing and analysis of forthcoming “big data.” **(C)** Definition of the comprehensive roles of miRNAs across diverse vascular cell types to explore further the RNA-dependent control of vascular crosstalk. **(D)** Establishment of rigorous standards for robust and independent validation of miRNA activity in PH. Illustrated by Mao Miyamoto.

to study targets entirely unknown to this disease. New methodology to isolate diseased cell types from living PH patients (94) will also ensure a substantial advance toward an era of personalized miRNA medicine in PH.

Second, with a global investment in high-throughput data, our computational techniques will also need to evolve, particularly for ascertaining a comprehensive view of miRNA-target effector network architecture and kinetics. For example, most miRNAs studied to date in PH were identified by their differential disease expression. However, in a disease state such as PH, we now know that a given miRNA can shift its target gene pool and functions, without altering its own expression (i.e., via alterations of target gene stoichiometry, alterations of A-U nucleotide editing, etc.; ref. 95), as in the case of miR-21 (28). Thus, rather than merely attaining differential gene expression lists, the delineation of coexpression networks of miRNAs with their targets could offer otherwise hidden insights embedded in a seemingly endless array of data. Statistical algorithms to generate differential dependency networks such as Evaluation of Dependency Differential-itY (EDDY) (96) have been implemented effectively in cancer data sets and could reveal insights into the individualized responses of these molecules in PH progression or with therapeutic interventions. To do so, however, a much greater financial and intellectual impetus for collaboration will be necessary to bring together computational scientists, experimentalists, and PH clinicians — groups that have had little previous opportunity for establishing long-lasting working relationships.

Third, few attempts have been made to analyze cell type-specific actions of miRNAs in the pulmonary vasculature and failing RV. This likely stems from the fact that most vascular miRNAs identified as relevant to PH to date are ubiquitously expressed in multiple cell types (22). Thus, many miRNAs have been associated with PH pathophenotypes that are shared among multiple diseased pulmonary vascular cell types (i.e., miR-130/301 affecting proliferation; ref. 66). Alternatively, for some miRNAs, their relevance to PH has thus far only been established in one cell type (i.e., miR-124 in fibroblasts; ref. 74) but may nonetheless be active in other cell types. An interrogation of miRNA actions across cell types may define another layer of interconnectivity. For instance, it may reveal a wide network of direct paracrine or endocrine messaging among cells that rely upon cellular release and uptake of miRNAs. Indeed, recent studies have suggested roles for cell-free miRNAs taken up by pulmonary vascular cell types in response to hypoxia (miR-210) (56) and in remodeled pulmonary vessels (miR-143) (81). Thus, a more comprehensive endeavor to differentiate cell type-specific functions of miRNAs may offer an entirely new appreciation of pulmonary vascular crosstalk.

Fourth, maintaining rigor in miRNA science is especially important, as *-omics* analyses reach a zenith. Considerations include the ultra-sensitive but differing modalities of quantifying miRNA expression (often leading to false-positive detection of miRNAs in contaminated reagents; ref. 97). In part, these issues may result from the dynamic and context-specific nature of miRNA expression and function coupled with the often modest and “fine tuning” effects on their direct targets. Although there exists no fool-proof solution, rigor can be strengthened at the level of the researcher and peer reviewer by requiring multiple independent modalities of assessing miRNA expression and function. For example, while global study of miRNAs by RNA sequencing continues to become less costly (98), results from this modality still should be verified by a separate method of RNA detection (i.e., RT-PCR, Northern blot). In cases where human tissue or plasma is studied, investigators should strive to include both a discovery and validation cohort, if possible. In screening for miRNA target genes, the use of computational algorithms predicting miRNA targets, which have varying degrees of false-positive and false-negative predictive rates (99), can be strengthened when coupled with biochemical screening assays such as RNA-CLIP (AGO-miRNA immunoprecipitation) (100). Finally, both gain- and loss-of-function assays to overexpress and inhibit miRNA function *in vitro* and *in vivo* are extremely valuable when utilized in tandem for assessing the robustness and biologic relevance of a given miRNA. More effective and less cumbersome modalities to modulate cohorts of miRNAs in the same cells or tissues simultaneously will be particularly useful in interrogating the complex interactions of multiple miRNAs. In sum, more comprehensive experimental methodology will be crucial for determining which miRNAs may be developed further for effective diagnostics and therapeutics in PH.

Implementing complex miRNA biology into diagnostics and therapeutics

miRNA diagnostics. The ability to identify stabilized miRNAs in the plasma and extracellular space has offered the potential to leverage their quantitation for diagnostic possibilities in PH. Cell-free, plasma-based miRNAs are packaged either with AGO2 in microvesicles (101) or as free-floating RNA-protein complexes (102), as we reported (56). Some cell-free miRNAs may be released and/or taken up by diseased vascular or cardiac tissue and may be handled distinctly from endogenous miRNAs (103). This may allow for effective communication among immune cells and vascular endothelium (104, 105) and enable pulmonary vascular crosstalk in diseased pulmonary vessels (miR-143) (81). Other miRNAs have been reported as differentially expressed in the circulating plasma of PH patients, including miR-150 (106), miR-23a (107), miR-26a (108), and miR-125a (109), but their biology is less understood. Alteration of entire cohorts of plasma miRNAs has been reported in PH (107, 110), as well as CTEPH (86). While these and other miRNAs are considered as prospective biomarkers in PH (Table 3), numerous pitfalls remain for their development as robust diagnostic tests, including issues with interindividual variability, reproducibility among patient cohorts, the tissue source of circulating RNAs, appropriate modality of testing, and the complexity of validating a true signature of miRNAs rather than a single biomarker.

Beyond plasma, quantification of intracellular miRNA content in blood cells may mitigate some of the variability seen in plasma sampling. While such strategies clearly are not reflective of miRNA-based processes occurring in vascular tissue directly, our advancing understanding of inflammatory activation in PH (111, 112) indicates the relevance of tracking blood-borne inflammatory cells and the miRNAs that control their function.

Finally, rather than relying solely on miRNA expression, diagnostic miRNA testing could be enhanced by further definition of genomic polymorphisms or variants that contribute to PH. Nucleotide variants may localize to a miRNA sequence, the miRNA biogenesis machinery, or to the binding sites of target mRNAs.

Table 3. miRNAs as potential circulating biomarkers of PH

S. No.	miRNA(s)	Experimental description	Expression	Reference
1	miR-150	Samples: PH and normal patients plasma ($n = 8$); followed by validation in two different cohorts of $n = 145$ and 30 . Method: miRNA expression array	Downregulated	(106)
2	miR-23a	Samples: 12 idiopathic PH patients and 10 healthy controls Method: miRNA expression array	Upregulated	(107)
3	miR-26a	Samples: 4 treatment naive idiopathic PH patients and 3 healthy controls. Later validated in 14 PH patients (6 idiopathic and 8 associated) and 13 controls Method: miRNA expression array	Downregulated	(108)
4	miR-1, -26a, -29c, -34b, -451, -1246, -130a, -133b, -191, -204, -208b	Samples: 8 controls, 15 moderate, and 16 severe PH, as assessed by mean pulmonary artery pressure. Method: miRNA expression array	Downregulated: miR-1, -26a, -29c, -34b, -451, -1246; Upregulated: miR-130a, -133b, -191, -204, -208b	(110)

Such studies have revealed interesting connections of PH to polymorphisms affecting miR-146a processing (113), the 3' UTR of the epithelial growth factor receptor (114), and the interaction between miR-759 and the fibrinogen α gene (87). As genomic sequencing costs continue to decrease, it may be possible to overlap genomic miRNA-relevant polymorphisms with miRNA expression levels in order to provide more specificity for these diagnostic and prognostic endeavors.

miRNA therapeutics. Mirroring the emerging trends of RNA-based diagnostics, the prospects of miRNA-specific therapies to augment or knockdown miRNAs are at their inception. Although some miRNAs have been proposed as potential therapeutic targets in PH (19), their clinical potential remains an open question. Primarily, miRNA-based therapeutic strategies have concentrated on the development of stabilized oligonucleotide mimics or inhibitors, but these have yet to be explored in clinical trials for PH. Oligonucleotide therapies have entered the clinical stage for a number of diseases beyond PH (115–117). Regarding miRNAs, currently, only one antisense oligonucleotide miRNA inhibitor, targeting miR-122 in the liver for hepatitis C infection, has undergone a successful phase 2a clinical trial (117). However, a phase 1 clinical trial using miR-34a mimic oligonucleotides for liver cancer (<http://clinicaltrials.gov/ct2/show/NCT01829971>) was recently halted for several immune-related adverse events.

From a technical perspective, delivery and packaging of oligonucleotides to ensure delivery to the pulmonary vasculature create challenges. Multiple effective routes of administration have been used in PH rodents, including i.v., inhalational, s.c., and i.p. injections. Historically, adenoviral or lentiviral vectors have been proposed for consistent and long-lasting expression in vivo (118, 119), but this approach would require transgenic rather than oligonucleotide-based delivery. For oligonucleotides, conjugation with various agents such as cholesterol, TLR ligands, integrin-specific antibodies, or antibody Fab fragment conjugation have been tested to assist in targeted uptake (120). RNA or DNA binding peptides have also been used in place of protein for linking oligonucleotides to antibodies (120). More recently, the use of polymer-based nanoparticles (55, 121) or packaging in microvesicles (122) has been explored in pharmacologic manipulations of miRNAs in PH. While some tissue specificity was observed, delivery only to the diseased pulmonary vasculature has yet to be achieved in a robust manner. Moreover, targeted delivery may come with a price of unwanted activation of the immune system. Thus, particularly for PH, the desired combinations of oligonucleotide packaging, modifications, and specificity of delivery have been elusive to achieve maximal stability, uptake, and targeting, while minimizing toxicity, immunological activation, and clearance from the system.

Perhaps more importantly, a primary conceptual obstacle includes the choice(s) of which miRNAs to target in PH. While the pleiotropy of several PH-relevant miRNAs has been touted as an advantage for developing broadly acting therapies, disadvantages have emerged regarding the toxic side effects of any miRNA-specific drug and the context-specific activities of these miRNAs across different tissue or cell types. Furthermore, given the overlapping and sometimes redundant biology of miRNAs in PH, it is becoming increasingly likely that a single miRNA target may be insufficient in preventing, improving, or regressing the overall manifestations of PH. Rather, a targeting of a combination of convergent miRNAs, as well as their downstream target genes, may be much more effective. Realization of this strategy, however,

would require a systematic mechanism to screen, either computationally or experimentally, such combinations of miRNA-target genes — technology that often is not available in purely academic settings. Thus, successful development of miRNA-based therapeutics in PH will necessitate combining an understanding of the higher-order regulatory hierarchy of miRNA biology with a deeper collaboration of academic investigators with federal, pharma, and biotechnology partners.

Conclusions

The past 15 years have yielded a wealth of scientific insights into the complex biology of ncRNAs and miRNAs in PH and beyond. This growth of knowledge has molded our collective appreciation of the daunting complexity of RNA-based regulation of gene expression in this disease. Thus, in some ways, this complexity has brought more confusion to the precise organized structure of miRNA-based mechanisms that drive disease. To overcome those deficiencies, the next phase of research and discovery will necessitate a pipeline of systematic endeavors designed to catalog and identify the hierarchy of activity inherent in these molecular networks. If successful, that next level of insight should further invigorate interest from academia, federal, and industry partners to pursue the collaborative development of more effective RNA-based diagnostics and therapeutics, based on such systems-level understanding of this disease.

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