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MicroRNA in the Diseased Pulmonary Vasculature: Implications for the Basic Scientist and Clinician

Richard C. Jin, MD[1], Pil-Ki Min, MD[2], Stephen Y. Chan, MD[1]

[1]*Division of Cardiovascular Medicine, Department of Medicine, Brigham and Women's Hospital, Boston, USA;*
[2]*Cardiology Division, Heart Center, Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul, Korea*

ABSTRACT

Since the first descriptions of their active functions more than ten years ago, small non-coding RNA species termed microRNA (miRNA) have emerged as essential regulators in a broad range of adaptive and maladaptive cellular processes. With an exceptionally rapid pace of discovery in this field, the dysregulation of many individual miRNAs has been implicated in the development and progression of various cardiovascular diseases. MiRNA are also expected to play crucial regulatory roles in the progression of pulmonary vascular diseases such as pulmonary hypertension (PH), yet direct insights in this field are only just emerging. This review will provide an overview of pulmonary hypertension and its molecular mechanisms, tailored for both basic scientists studying pulmonary vascular biology and physicians who manage PH in their clinical practice. We will describe the pathobiology of pulmonary hypertension and mechanisms of action of miRNA relevant to this disease. Moreover, we will summarize the potential roles of miRNA as biomarkers and therapeutic targets as well as future strategies for defining the cooperative actions of these powerful effectors in pulmonary vascular disease.

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Key Words: MicroRNAs; Pulmonary hypertension; Anoxia; Vascular diseases

Introduction

The most well-studied sequences in the human genome are those of protein-coding genes, with the coding exons of these genes comprising approximately 1.5% to 2% of the genome.[1)] Recently, epigenetic alterations of the genome (e.g., DNA methylation) and the transcriptome (e.g., repression by non-coding RNA) have been recognized as powerful and alternative gene regulatory mechanisms, and it has become apparent that the non-protein-coding portion of the genome plays a significant role in normal development and pathophysiology of human disease.[2)] In particular, microRNA (miRNA) are small, evolutionarily conserved, non-protein coding RNA molecules that have been found to carry especially pleiotropic and ubiquitous actions in gene regulation and human pathogenesis.

At present, greater than 1,400 distinct miRNA species have been identified or predicted in human cells.[3)] Moreover, roughly 50% and 60% of all mammalian messenger RNA (mRNA) transcripts are estimated to be di-

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Correspondence to: Stephen Y. Chan, MD
Address: Division of Cardiovascular Medicine, Department of Medicine, Brigham and Women's Hospital, New Research Building, Room 630N, 77 Avenue Louis Pasteur, Boston, MA 02115, USA
Tel: +1-617-525-4844, Fax: +1-617-525-4830
E-mail: sychan@partners.org

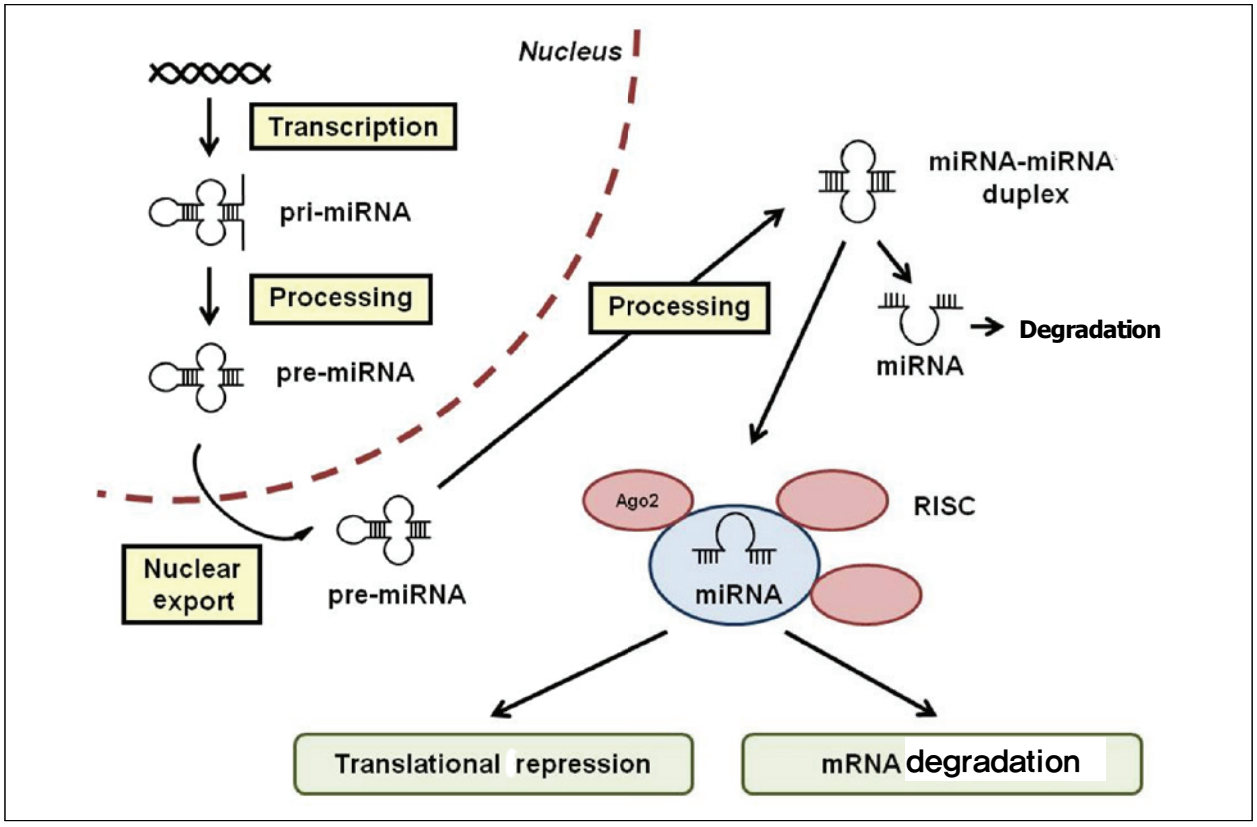

Fig. 1. MicroRNA (miRNA) biogenesis and mechanism of action. MiRNAs undergo several nuclear and cytosolic processing steps before maturation to a biologically active form (19–24 nt). After processing, the mature miRNA is incorporated into the RNA-induced silencing complex (RISC). Through binding to the complementary sites in the 3' untranslated region of their target genes, miRNA promote down-regulation of the protein synthesis via translational repression or messenger RNA (mRNA) degradation.

rect targets of such miRNA.[4,5] MiRNAs are genomically encoded either as single genes ("intergenic"), as a polycistronic family, or as a gene embedded in an intron of a protein-coding gene ("intronic"). Upon transcription to an approximately 1,000 nucleotide precursor (primary miRNA or pri-miRNA), several nuclear and cytosolic processing steps occur leading to maturation to a mature, biologically active double stranded miRNA duplex, consisting of a prototypical active strand that binds target transcripts for gene repression and an "inactive" strand (Fig. 1). The mature miRNA is incorporated into the RNA-induced silencing complex including the RNA-binding protein Argonaute 2 which is instrumental in mediating the recognition and binding of mature miRNA with its target mRNA transcript(s).[6] At the 5' end of each miRNA strand exists a "seed sequence" (at positions 2–8) which is an important determinant for complementary strand target sequence binding, typically at the 3' untranslated region (3'UTR) of the target mRNA transcript. The remainder of the miRNA sequence typically binds imperfectly, creating bulges and mismatches in the miRNA:mRNA heteroduplex.[7,8] Therefore, a single miRNA sequence can target hundreds of mRNAs by low miRNA-target complementarity.[9] Furthermore, more than one miRNA can cooperatively bind to the same 3'UTR and, thus, affect a stronger action.[10] As a result of such binding, a miRNA can promote the specific down-regulation of a cadre of mRNA targets, through translational

repression and/or mRNA degradation.[11,12]

In experiments involving both cultured cardiovascular cell types and rodent models of cardiovascular disease, specific gain- and loss-of-function studies of individual miRNA have elucidated their distinct roles in cardiovascular development and physiological function. Overall, miRNA tend to act by "fine tuning" the regulation of gene expression, disruption of which can have dramatic phenotypic consequences especially under stressful conditions.[13] Accordingly, multiple miRNA have been implicated in cardiomyocyte biology such as those involved in myocardial fibrosis, infarction, and hypertrophy (Fig. 2A).[14,15] Several miRNA have also been identified as modulators of systemic vascular remodeling, such as atherosclerosis, vasomotor tone and hypertension, and neointimal formation (Fig. 2B).[16,17] However, the importance of miRNA in the pulmonary vasculature is just becoming defined. Here, we aim to review our basic understanding of the molecular mechanisms important in pulmonary vascular diseases such as pulmonary hypertension with the intent to correlate the biology of miRNA with these processes. Consequently, we hope to emphasize the clinical importance of studying miRNA in this context, providing a platform for discovering new molecular mechanisms of pulmonary vascular disease, identifying novel miRNA biomarkers of disease, and potentially determining putative miRNA-based therapeutic targets to ameliorate, regress, or prevent pulmonary hypertension.

Pulmonary hypertension and its known molecular pathogenic mechanisms

1. Clinical definition & classification of pulmonary hypertension

Pulmonary hypertension (PH) is a hemodynamic state in which the pulmonary artery pressure is abnormally high and is clinically defined as a resting mean pulmonary arterial pressure (mPAP) > 25 mm Hg.[18] PH is a progressive disease of various etiologies that has a poor prognosis, ultimately leading to right ventricular failure,[19] multi-organ dysfunction, and often death. Based on the latest World Health Organization classification,[20] PH is classified into 5 categories based on presumed primary etiology: 1) pulmonary arterial hypertension (PAH), 2) PH owing to left heart disease, 3) PH owing to lung disease and/or hypoxia, 4) chronic thromboembolic PH, and 5) PH with unclear, multifactorial mechanisms.

Specifically, PAH (group 1) and hypoxia-induced PH (group 3) have historically been the focus of substantial study since the first standardized classification of PH was released. Consequently, most of our mechanistic insights into PH have originated from study of these groups. Specifically, PAH is defined as a resting mPAP > 25 mm Hg or exercise-induced mPAP > 35 mm Hg, accompanied by an elevated pulmonary vascular resistance (>3 Wood units), normal pulmonary capillary wedge pressure (<15 mm Hg), and absence of predisposing conditions such as left heart disease, hypoxic lung disease, and thromboembolic disease.[20] Currently, it comprises idiopathic PAH (IPAH) as well as secondary PAH derived from genetic or familial predisposition, drug- and toxin-induced PAH, flow-induced PAH associated with congenital heart disease and cardiac shunts, persistent PH of the newborn, pulmonary veno-occlusive disease, and pulmonary capillary hemangiomatosis.[20] On the other hand, hypoxia-induced PH is defined as a resting mPAP > 25 mm Hg accompanied by secondary diseases indicative of chronic hypoxic exposure, including chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, obstructive sleep apnea, and chronic high-altitude exposure. Despite some differences among these variants of PH, substantial overlap is also present at the molecular level,

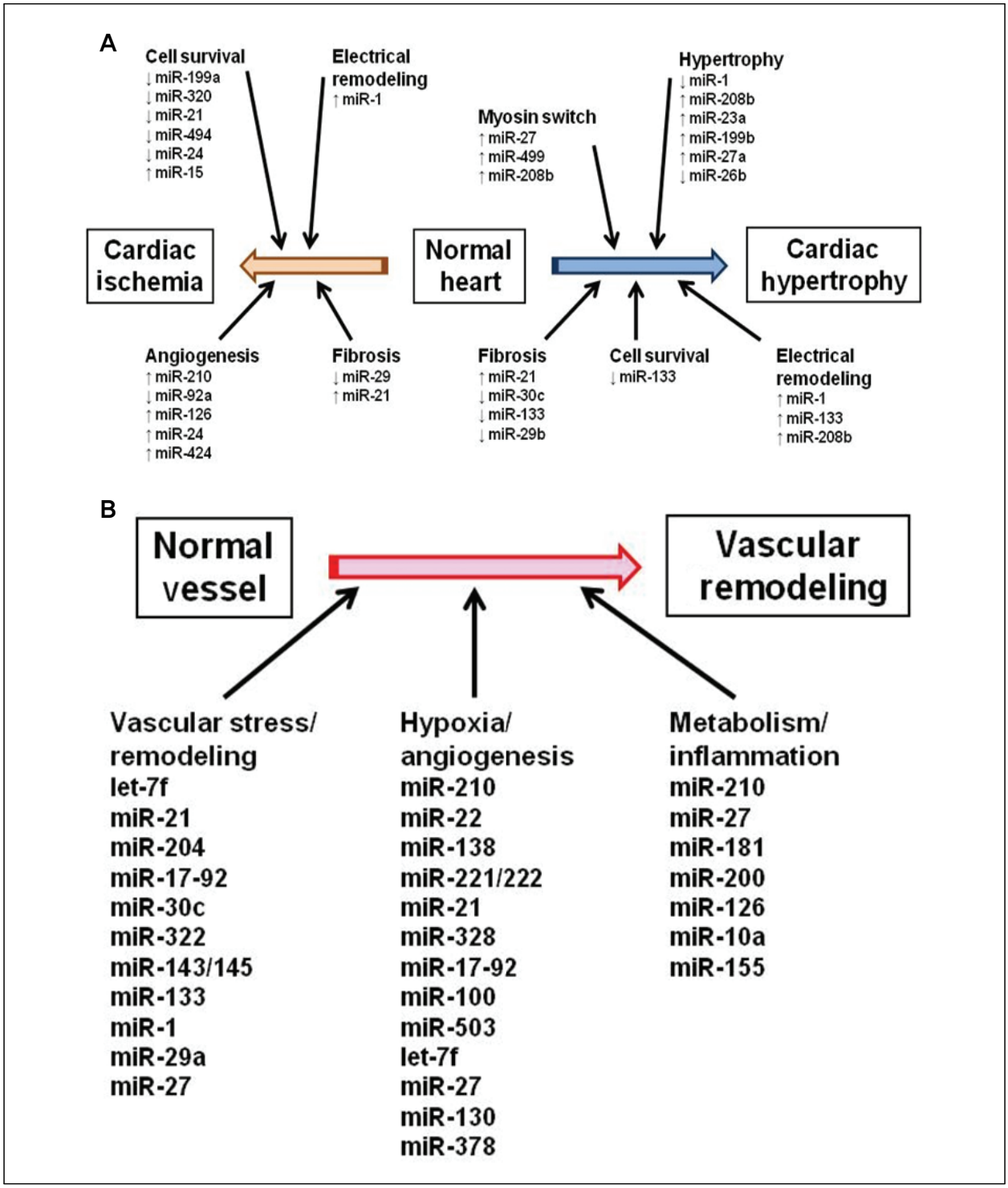

Fig. 2. Role of microRNAs (miRNAs) in cardiovascular disease. (A) Control of cardiomyocyte function by miRNA. The pathogenic events influencing cardiomyocytes in ischemic and hypertrophic diseases are closely controlled by multiple miRNA which regulate cell survival, apoptosis, angiogenesis, fibrosis, and remodeling.[14] (B) Control of vascular function by miRNA. Pathophenotypes in diseased vasculature are closely controlled by miRNA, which regulate vascular integrity and remodeling.[15,16] From White K, et al. Pulm Circ. 2012;2:278–90, with permission of Pulmonary Circulation.[17]

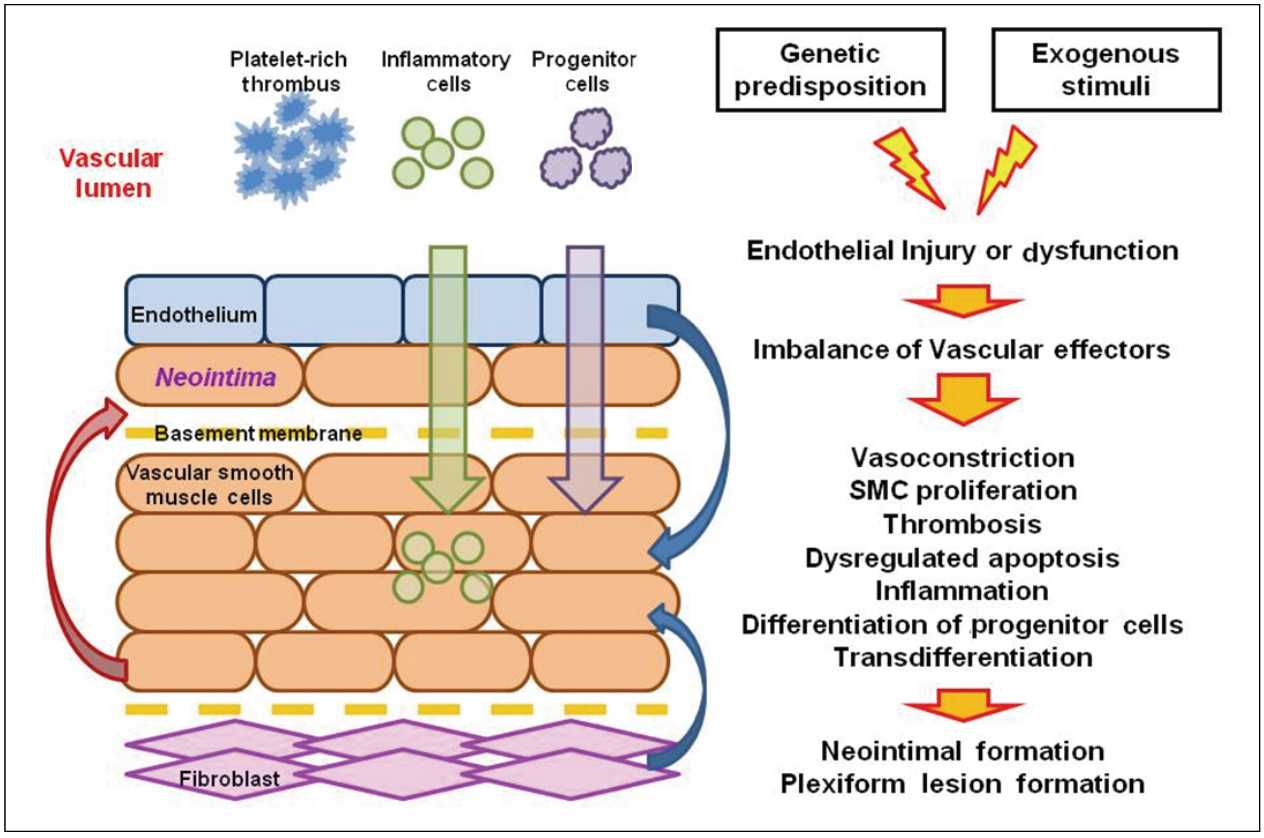

Fig. 3. Pathobiology of pulmonary hypertension. Multiple vascular cell types in the pulmonary arterial wall and pulmonary arterial circulation are involved in the pathobiology of pulmonary arterial hypertension (PAH). Vascular pathology in PAH is triggered by various genetic and environmental stimuli, and initial injury to the endothelium and/or adventitial fibroblasts may activate pathogenic signaling pathways. These result in an imbalance of secreted vascular effectors that cause excessive pulmonary vasoconstriction and abnormal vascular remodeling processes. Common histological features in PAH include intimal hyperplasia, medial hypertrophy, adventitial proliferation/fibrosis, occlusion of small arteries, thrombosis *in situ*, and infiltration of inflammatory cells (green arrow) or progenitor cells (purple arrow). Transdifferentiation of endothelial cells or fibroblasts to vascular smooth muscle cells may contribute as well (blue arrow). These vascular changes lead to the formation of a layer of "neointima" (red arrow) and, in some cases, plexiform lesions (From Chan SY, et al. J Mol Cell Cardiol. 2008;44:14–30, with permission of PubMed Central).[34]

with both disease types displaying pronounced imbalance of cell growth and cell death, leading to remodeling of the pulmonary vasculature as well as changes in vasomotor tone and thrombotic potential. It remains unproven if similar mechanisms of disease primarily contribute to the burden of vascular disease seen in groups 2, 4, and 5. However, the functional importance of these similarities in all groups has been suggested by clinical data demonstrating that pulmonary vasodilator therapy is effective across groups 1 to 4.[21] In this review, we will focus primarily on disease mechanisms directly applicable to these two groups, with possible extrapolation of those principles to other types of PH.

2. Histopathobiology of pulmonary hypertension

In general, various forms of PH and PAH are marked by a complex panvasculopathy affecting small pulmonary arterioles, marked by the dysregulation of multiple cell types and molecular pathways (Fig. 3). Our understanding of the underlying pathobiology of PH has become increasingly

complex as multiple genetic and molecular pathways have been identified.[22] Common histologic features in PH include intimal hyperplasia, medial hypertrophy, adventitial proliferation/fibrosis, occlusion of small arteries, thrombosis *in situ*, and infiltration of inflammatory/progenitor cells.[23] Later in disease, these changes can result in disordered angiogenesis and formation of pathognomonic complex vascular lesions, or so-called plexiform lesions.[24] This vascular pathology is triggered by various genetic and environmental stimuli, and multiple vascular cell types in the pulmonary artery and pulmonary arterial circulation contribute to the specific response to injury and the development of PH.[25] The initiating cell type remains unclear, but abnormalities in pulmonary endothelial cell and pulmonary artery smooth muscle cells as well as transdifferentiation among these two populations may primarily contribute to the development of PH.[26] Adventitial fibroblasts likely contribute as well, as they display increased proliferative capacity in PH, further contributing to vascular fibrosis. Inflammatory cells and activated platelets can predominate in end-stage PH, but our understanding is limited regarding the mechanistic role of these populations in disease progression. Finally, pulmonary arterial remodeling is associated with chronic inflammatory events and may promote the recruitment of circulating or resident progenitor cells, which in turn may dysregulate vascular proliferative capacity.[27,28]

3. Molecular mechanisms of pulmonary hypertension

Inherent in the complicated phenotypes that are observed as PH develops are the complex and overlapping molecular mechanisms that appear to be driving and/or are associated with these conditions. From a genetic perspective, mutations in the transforming growth factor-β (TGF-β) receptor superfamily, particularly the bone morphogenetic protein receptor type 2 (BMPR2) gene[29,30] have been genetically linked to PAH and likely play a causative role in the development of disease. Genetic abnormalities in serotonin signaling and transport have also been associated with PAH in human cohorts.[31] Alternative mechanisms involving complementary "modifier" genes could contribute to a genetic predisposition to PH, as suggested for single nucleotide polymorphisms in genes encoding for the voltage-gated potassium channel $K_V1.5$ and the transient receptor potential cation channel, subfamily C, member 6.[32] In addition to genetic predisposition, various physiologic, acquired, and/or exogenous stimuli modulate the development of PH. Some of these stimuli have been studied to a sufficient degree at the molecular level, while other factors carry much less defined mechanisms of action (Fig. 4). Downstream of the genetic and acquired triggers of PH, the histopathologic processes that predominate later stages of disease include vasoconstriction, smooth muscle cell and endothelial cell proliferation, and thrombosis.[33] Historically, it was thought that these processes are influenced primarily by a complex and dysregulated balance of vascular effectors controlling vasodilatation and vasoconstriction, growth suppressors and growth factors, and pro-versus anti-thrombotic mediators (Fig. 5). While these factors are certainly important in the overall disease process, it is now apparent that these effectors are subject to upstream, over-arching regulatory pathways that affect the action of multiple vasoactive molecules.[34] These include mitochondrial and metabolic dysregulation, alterations in potassium channel signaling and intracellular calcium handling, perturbations in tyrosine kinase and Rho kinase signaling, inflammatory stimuli, and extracellular matrix disturbances, among others.

MicroRNA and pulmonary vascular disease

Considering the complexity and overlapping nature of

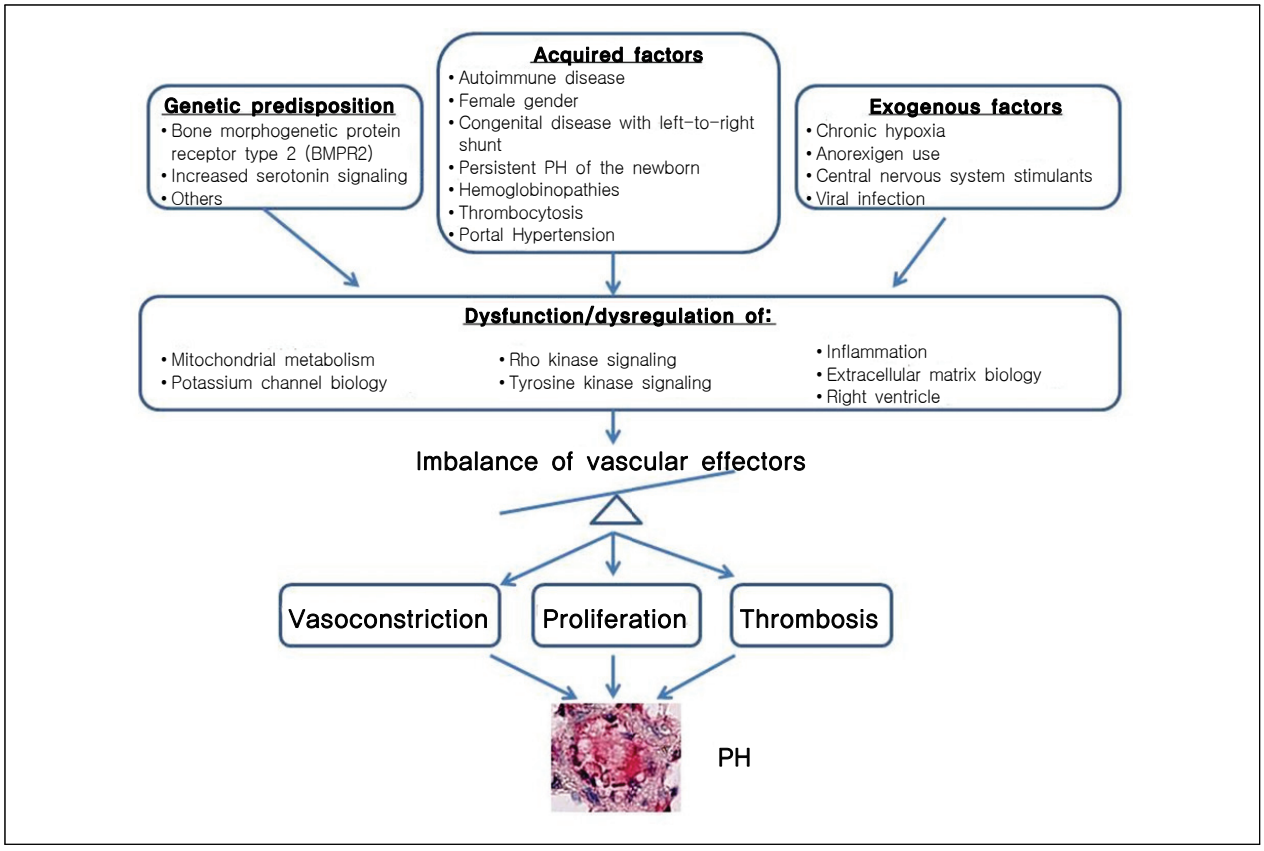

Fig. 4. Pathogenic mechanisms of pulmonary hypertension (PH). Complex, pathogenic mechanisms that connect genetic predisposition, acquired, and exogenous factors play a role in the downstream dysfunction/dysregulation metabolism and signaling pathways resulting in PH.

the molecular mechanisms important in PH along with the pleiotropic and ubiquitous modes of action of miRNA in mammalian tissue, it is reasonable to expect that miRNA carry substantial roles in modulating pulmonary vascular homeostasis and the development of pulmonary vascular diseases. However, unlike more expansive scientific literature describing the expression and actions of miRNA in other cardiovascular diseases, only a handful of miRNA thus far has been identified as direct modulators in PH. In part, this may be a reflection of the anatomic inaccessibility and difficulty in obtaining relevant disease tissue for study, especially as the frequency of lung transplantation for PH has been declining over the past decade. Nonetheless, a more substantial number of studies have been published recently that have identified a growing list of core miRNA critical in mediating these pulmonary vascular disease phenotypes.

1. MicroRNA related to upstream triggers of pulmonary hypertension and dysregulated in end-stage pulmonary hypertension

To begin to identify the functions of miRNA in PH, it has proven useful to relate specific miRNA to this disease if they carry already established functions related to upstream triggers of this disease. Stemming from the substantial studies of hypoxia-induced PH over the past two decades, the identification of hypoxia-responsive miRNA, termed as "hypoxamirs" by our group,[35] has offered a reasonable starting point. Hypoxia induces a complex set of adaptations that influence cellular survival and function in the pulmonary

Vascular effector	Effect on vasoconstriction	Effect on cell proliferation	Effect on thrombosis	Change in activity in PAH
Nitric oxide	↓	↑	↑	↑
Serotonin	↑	↑	↑	↑
Prostacyclin	↓	↑	↑	↑
Thromboxane A_2	↑	↑	↑	↑
Endothelin-1	↑	↑	—	↑
Vasoactive intestinal peptide	↓	↑	↑	↑
Peroxisome proliferator-activated receptor $-\gamma$	↓	↑	—	—

Fig. 5. Roles of vascular effectors in the pathogenesis of pulmonary arterial hypertension (PAH). Vascular effectors have been shown to have different roles in the pathogenesis of pulmonary hypertension (PH) (From Chan SY, et al. J Mol Cell Cardiol. 2008;44:14-30, with permission of PubMed Central).[34]

vasculature. In large part, these cellular adaptations are transcriptionally controlled by the hypoxia-inducible factors (HIFs). HIF consists of an unstable α subunit and a stable β subunit, and three HIF-α proteins have been described in higher metazoans: HIF-1α, HIF-2α, and HIF-3α.[36,37] Under hypoxia, HIF-α protein is not hydroxylated and is stabilized for heterodimerization with HIF-β. This heterodimer can bind specific sites in gene promoters termed hypoxia response elements, thereby inducing downstream transcription. HIF directly induce the transcription of more than 100 different genes, influencing a wide range of cellular processes. In aggregate, these events effectively reprogram the hypoxic cell for acute survival in a low oxygen environment.[38,39] Yet, such reprogramming is detrimental if chronically persistent. Consequently, HIF and its downstream targets have been implicated as crucial pathogenic factors across multiple clinical categories of PH, even in cases of PAH which are not directly associated with exogenous hypoxic exposure.[40] Although more than 100 hypoxamirs have been identified, most demonstrate modest induction, and are up-regulated only in certain cellular contexts or by HIF directly. Of these hypoxamirs, only a few have been defined as direct modulators of pulmonary vascular function and disease.

As well-established triggers of PH, inflammation and BMP pathway are also closely related with miRNA functions. Moreover, there is a considerable overlap among miRNAs in these categories and hypoxamirs. Although the direct actions of many of these miRNAs are still unknown, there is growing evidence that TGF-β and BMP superfamily signaling are intricately related to miRNA biology at multiple mechanistic levels?via regulating miRNA expression at the transcriptional and post-transcriptional levels and via inducing negative regulatory feedback loops through multiple miRNA with predicted and increasingly validated roles in repressing expression of TGF/BMP signaling components. Considering the fact that BMPR2 haploinsufficiency represents a major

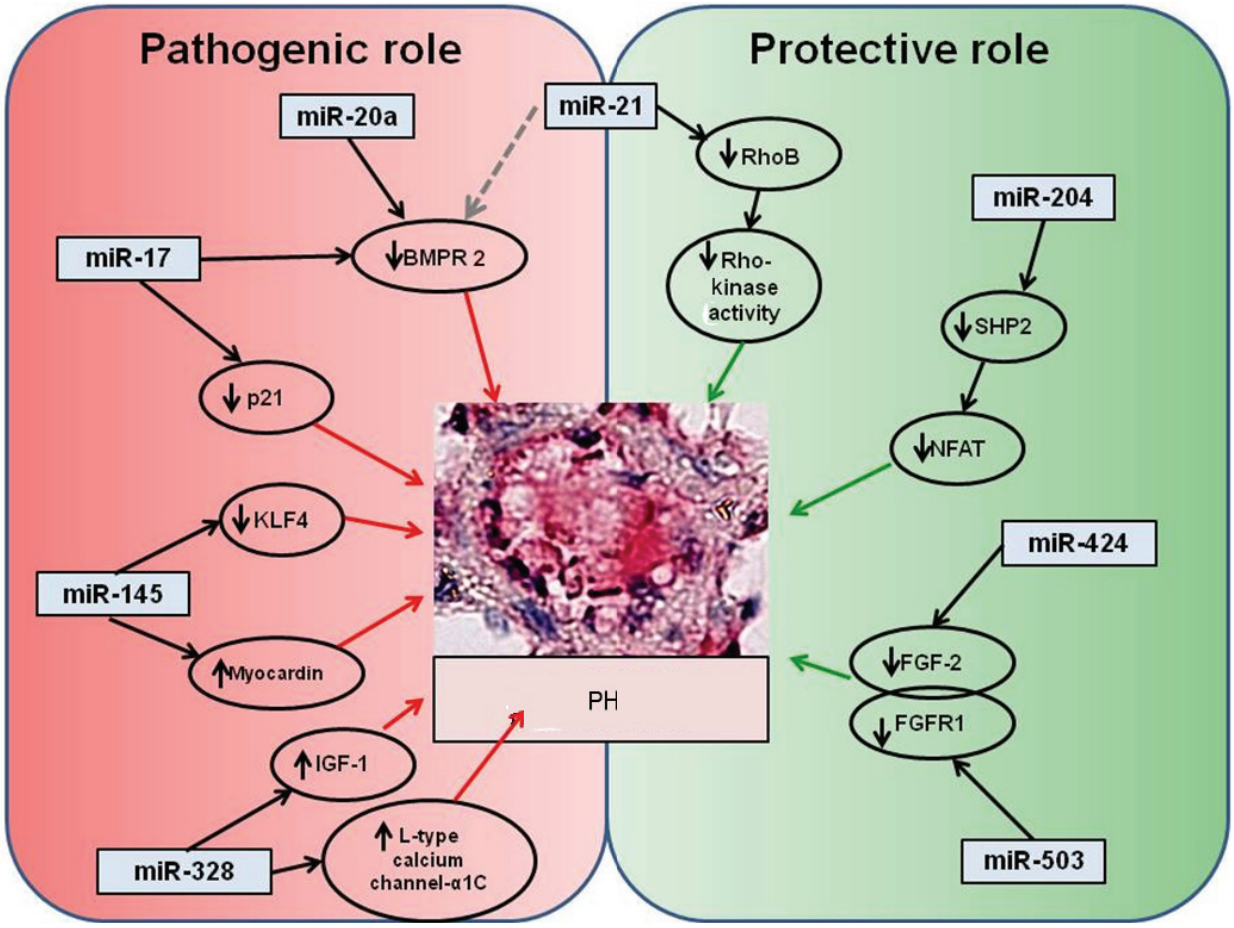

Fig. 6. Validated actions of microRNA (miRNA) in pulmonary hypertension (PH). MiRNA are listed that carry confirmed functions in controlling PH in rodents and humans, as categorized by protective or pathogenic roles. Down-regulation of miR-145 protects against the development of pulmonary arterial hypertension (PAH), possibly through attenuation of myocardin expression.[49] MiR-328 directly represses L-type calcium channel-α1C expression, resulting in reduction of pulmonary vasoconstrictive properties. Down-regulation of insulin growth factor 1 receptor may also contribute to pulmonary artery smooth muscle cell apoptosis.[50] MiR-21 down-regulates RhoB and Rho-kinase activity to protect against mechanisms driving PAH.[41] An antagomir directed against miR-20a restored functional bone morphogenetic protein receptor type 2 (BMPR2) signaling preventing PAH.[48] For miR-17, the BMPR2 transcript is a predicted and validated target in cultured vascular cells.[47] MiR-204 directly targets Src homology 2 domain-containing tyrosine phosphatase (SHP2) expression that inhibits nuclear factor of activated T cells (NFAT) expression playing a protective role against PAH.[44] Down-regulation of miR-424 and miR-503 in PAH is associated with increased fibroblast growth factor 2 (FGF2) and fibroblast growth factor receptor 1 (FGFR1) expression.[51] KLF4, Kruppel-like factor-4; IGF-1, insulin-like growth factor.

genetic predisposing risk factor for development of PAH in hereditary and familial cases, such a mechanistic connection may be an especially fruitful focus for research in PH. Yet, because of the sheer number of miRNA directly implicated in such TGF/BMP signaling loops, it is unlikely that a single miRNA alone serves as a predominant regulator of such signaling. Rather, we would expect that the coordinated actions of multiple related miRNA may synergize to affect a final pathophenotypic shift in PH. Yet, these principles remain to be confirmed *in vivo*.

Confidence that any of these miRNA may influence PH has been bolstered by overlap with the results of recent

screens that identify miRNA that are dysregulated in PH *in vivo*. On one hand, we have designed an *in silico* computational network-based screen to rank a group of 29 miRNA with the highest likelihood of influencing the PH phenotype, based on the proportion of their direct targets on a network of genes important in this disease.[41] Importantly, a large proportion of these miRNA have been implicated previously in hypoxic, inflammatory, and TGF/BMP signaling. On the other hand, several high-throughput expression screens of miRNA dysregulated in PH have been reported recently. One early study analyzed 350 miRNAs in whole lung homogenates derived from rodent models of PH.[42] Based on the level of dysregulation in one or multiple animal models, miR-322, miR-451, miR-21, miR-22, miR-30c, let-7f, and let-7a were further validated in both rodent and human examples of disease. A follow-up study subsequently reported alterations in miR-21 and miR-451, accompanied by dysregulation of miR-210 and miR-144.[43] Yet, because they represent the net changes of a combination of pulmonary cell types, these results do not necessarily reflect the exact miRNA profiles in the specific diseased pulmonary vessels. A high-throughput miRNA screen method was utilized by Courboulin et al.[44] in studying cultured ex vivo pulmonary arterial smooth muscle cells (PASMCs) obtained from patients suffering from IPAH as compared with healthy individuals. Of those found to be dynamically altered in IPAH-derived PASMCs, specific expression profiles were confirmed in lung biopsies derived from IPAH patients. In total, six miRNA were found to be consistently up-regulated in disease, including miR-138, miR-367, miR-276, miR-302b, miR-145, and miR-450a, while miR-204 was the only miRNA exhibiting substantial down-regulation. Recently, Bockmeyer et al.[45] also reported the *in situ* staining of specific miRNA in plexiform lesions in human PH lung. From the study of 12 PH patient samples as compared with 8 healthy controls, an up-regulation of miR-21 along with miR-126 was noted in plexiform lesions as compared with a down-regulation of miR-204 and miR-143/145. Importantly, the mechanistic significance of a few of these miRNA has been characterized further, but the putative importance of other dysregulated miRNA remains to be defined.

2. Validated microRNA controlling pulmonary hypertension

Based on the above screening strategies, a handful of miRNA have been mechanistically validated in their roles controlling PH in rodents and human *in vivo* (Fig. 6). Based on the previously described high-throughput screen of diseased human PASMCs cultured *ex vivo* demonstrating a down-regulation of miR-204, miR-204 inhibition was found to incite a hyper-proliferative and anti-apoptotic cellular phenotype as well as frank PH in a rodent model via the miR-204-dependent regulation of SHP2, Src activity, and downstream metabolic pathways.[44] Separately, based on the previously described network-based approach, our group confirmed that miR-21 is up-regulated in examples of mouse and human PH by hypoxia, inflammatory cytokines, and BMPR2-dependent signaling, thus leading to a down-regulation of its direct target RhoB, Rho kinase activity, and PH *in vivo*.[41] As induced by the interleukin-6/signal transducer and activator of transcription 3 inflammatory pathway, the miR-17-92 cluster can directly target BMPR2 in pulmonary vascular cell types.[46] Furthermore, it was recently demonstrated that a miR-17 inhibitor improved heart and lung functions in the PH mouse and rat models.[47] Similarly, an inhibitor of miR-20a reduced PH in hypoxic mice, potentially through a mechanism involving the rescue of BMPR2 expression.[48] More recently, the smooth muscle-specific miR-145 was found to induce PH *in vivo* presumably

through its down-regulation of multiple targets that influence the contractile phenotype of diseased PASMCs.[49] Additionally, by virtue of its hypoxic induction, the hypoxamir miR-328 has been implicated in PH via its repressive effects on L-type Ca(2+) channel genes and the insulin growth factor 1 receptor, subsequently driving PASMC apoptosis.[50] Finally, it was recently reported that apelin deficiency drives a down-regulation of miR-424 and miR-503 in pulmonary arterial endothelial cells in PH, thus driving a de-repression of the direct targets fibroblast growth factor 2 and its receptor and thus affecting pulmonary vascular proliferative capacity.[51] Validation of further causative actions of miRNA in this disease is expected to exponentially increase over next years, especially as improvements in both computational modeling and *in vivo* manipulation of multiple miRNA become optimized.

Further clinical implications of microRNA biology

1. Circulating microRNA and possible roles in pulmonary hypertension

Beyond the canonical intracellular actions of miRNA in the pulmonary vasculature itself, extracellular forms of miRNA may also play an important role in the regulation of pulmonary vascular function. Recently, extracellular miRNA have been detected in the bloodstream and circulate in a remarkably stable form.[52-54] Increasing evidence suggests that secreted miRNAs are protected from degradation in several different mechanisms. These mechanisms include storage of miRNAs in microparticles (exosomes, microvesicles, and apoptotic bodies),[55,56] protein complexes (Argonaute2),[57] or lipoprotein complexes (high-density lipoprotein).[58] It has also been suggested that they are released passively as a by-products of dead cells.[59] Only a handful of circulating miRNA have been identified in PH. For example, down-regulation of plasma miR-21 has been reported in blood from patients with idiopathic PAH.[42] Furthermore, it has been demonstrated that plasma miR-150 levels are down-regulated, and reduction of circulating miR-150 correlates with poor survival in patients with PAH.[60] Although their exact roles in PH pathogenesis are unclear, recent studies suggest that circulating miRNAs might play a role as mediators of cell-to-cell communication.[58,61-63] Thus, it is possible that miRNA may act as unique molecular messengers in PH, enabling communication between the disease pulmonary vasculature and other tissues. Furthermore, due to their remarkable stability in the circulation, miRNAs have been studied for their potential role as diagnostic and/or prognostic biomarkers in cardiovascular diseases including myocardial infarction, heart failure, peripheral artery disease, hypertension, and diabetes.[64-66] Comprehensive analysis of circulating miRNA in PH has not yet been completed and may demonstrate potential for diagnostic use, but these results may vary greatly on the location of plasma withdrawal (venous, arterial, or pulmonary arterial location) as a reflection of the originating tissue source of such circulating miRNA.

2. MicroRNA as therapeutic targets in pulmonary hypertension

As potent and crucial regulators in a wide variety of diseases, miRNAs have been also emerged as novel therapeutic targets but have yet to be substantially studied in PH. Currently the most widely effective approach to inhibit miRNA *in vivo* is the use of antisense oligonucleotide inhibitors (antagomirs or antimiRs). To increase the stability of miRNA inhibitors and allow their entry into mammalian cells without the need for additional transfection reagent, further chemical modification can be added, such as locked nucleic acid oligonucleotides, polylysine-conjugated peptide nucleic acids, and cholester-

ol-conjugated moieties.[67,68] Such inhibitors typically carry the complimentary reverse sequence of the active strand of a specific mature miRNA. This approach relieves the inhibition of the target genes by the miRNA and leads to the increase of expression of the target genes.[69] The first mammalian *in vivo* study used an antagomir to inhibit miR-122 and reported reduction of the level of the miRNA of interest and up-regulation of target genes.[70] Studies using antagomirs against miR-133 or miR-21 have been demonstrated that cardiac miRNAs also can be targeted with this approach.[71,72] Recently, in rodent models of PH, hemodynamic and histologic parameters of PH have been modulated by various miRNA inhibitors, including miR-21,[41,43] miR-17,[47] miR-20a,[48] miR-145,[49] and miR-424/503.[51] Alternatively, in circumstances when miRNAs are down-regulated in the disease state to induce disease, a different approach is needed for pathophenotypic reversal. To increase the active concentration of a specific miRNA, one successful approach has entailed the use of synthetic RNA duplexes designed to mimic the native miRNA (miR mimics). In the monocrotaline-induced PAH rat model, nebulization of miR-204 mimics was reported to reduce disease severity significantly.[44]

Although miRNA-based therapy continues to be the subject of substantial investigation in both the academic and biotechnology sectors, a number of challenges remain. Antisense technology has the potential to induce dose-dependent off-target effects, especially in contexts that entail high concentrations necessary for vascular delivery. Thus far, the ability to specifically deliver inhibitors to the pulmonary vasculature has yet to be realized without affecting other tissues. The advent of nanoparticle-based therapeutics may further advance the applicability of miRNA inhibition in PH. Biodegradable polymer nanoparticles represent a means to effectively deliver miRNA inhibitors, while minimizing the drawbacks of chemical modification that include systemic sequestration in the liver[70] and much higher doses required to achieve efficacy.[73,74] Furthermore, by utilizing a technique similar to Cheng et al.,[75] it may be possible to engineer nanoparticles carrying miRNA inhibitors with cell-penetrating peptides that may specifically target absorption across the airway and into the pulmonary vasculature. Finally, due to recent appreciation that a single miRNA may target many genes or pathways and multiple miRNAs may affect the same target, miR-based therapeutics may encounter substantial difficulty in tailoring a specific effect on a single target to influence disease phenotype *in vivo*. More effective miRNA-based strategies may necessitate the therapeutic targeting of multiple miRNA that work via the same network of targets to enforce a particular pathophenotype. Yet, challenges still remain even to identify rigorously the networks of those miRNA and their targets that carry the most robust actions in the control of PH *in vivo*.

Conclusion

In summary, as more miRNA targets are identified, we anticipate a substantial increase in our understanding of the molecular actions of various miRNA in PH. Given their pleiotropic actions in modulating multiple gene targets, we suspect that many of these may even serve as central molecular "lynchpins" connecting diverse upstream disease triggers to a common pathologic pulmonary vascular result. We predict rapid advances in this nascent field of study, including improved methods to integrate network and systems biology with evolving technologies in cell culture and *in vivo* experimentation with miRNA. As a result, our discovery process will be further accelerated in order to identify more comprehensively all relevant miRNA important in this highly morbid condition, with the ultimate goal of rapidly translating these insights and technology to possible regression, reversal, or prevention of PH.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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