MicroRNAs-control of essential genes: Implications for pulmonary vascular disease

Sachindra R. Joshi, Jared M. McLendon, Brian S. Comer, and William T. Gerthoffer

Department of Biochemistry and Molecular Biology, University of South Alabama, Mobile, Alabama, USA

ABSTRACT

During normal lung development and in lung diseases structural cells in the lungs adapt to permit changes in lung function. Fibroblasts, myofibroblasts, smooth muscle, epithelial cells, and various progenitor cells can all undergo phenotypic modulation. In the pulmonary vasculature occlusive vascular lesions that occur in severe pulmonary arterial hypertension are multifocal, polyclonal lesions containing cells presumed to have undergone phenotypic transition resulting in altered proliferation, cell lifespan or contractility. Dynamic changes in gene expression and protein composition that underlie processes responsible for such cellular plasticity are not fully defined. Advances in molecular biology have shown that multiple classes of ribonucleic acid (RNA) collaborate to establish the set of proteins expressed in a cell. Both coding Messenger Ribonucleic acid (mRNA) and small noncoding RNAs (miRNA) act via multiple parallel signaling pathways to regulate transcription, mRNA processing, mRNA stability, translation and possibly protein lifespan. Rapid progress has been made in describing dynamic features of miRNA expression and miRNA function in some vascular tissues. However posttranscriptional gene silencing by microRNA-mediated mRNA degradation and translational blockade is not as well defined in the pulmonary vasculature. Recent progress in defining miRNAs that modulate vascular cell phenotypes is reviewed to illustrate both functional and therapeutic significance of small noncoding RNAs in pulmonary arterial hypertension.

Key Words: hypertension, Krueppel-like factor 4, myocardin, smooth muscle, translation, vascular remodeling, vascular injury

INTRODUCTION

During development of the airways and the pulmonary circulation, multiple cell types adapt to changing chemical and physical signals to establish appropriate lung structure and function. Progenitor cells of various vascular cell types proliferate, migrate and reorient themselves to form nascent vessels and airways that eventually mature to the adult phenotype. During disease development there are also adaptive changes in function and structure, some of which are maladaptive and some of which oppose the disease process. Functional adaptations are manifest in the pulmonary circulation in pulmonary hypertension syndromes as hypercontractility.

Structural adaptations are manifest as vascular pruning, medial thickening, leukocyte invasion and development of a variety of occlusive lesions. The initial triggers or stimuli for these remodeling events are under intense investigation, as are the pathways and proteins altered during pulmonary diseases. Structural studies suggest multiple cell types including lung fibroblasts, myofibroblasts, smooth muscle, epithelial and progenitor cells all undergo varying degrees of phenotypic modulation during disease development. Blood vessel remodeling events include matrix remodeling, secretion of numerous cell signaling molecules, cell and tissue hypertrophy, and hyperplasia. In all hollow organs, including pulmonary blood vessels, smooth muscle cells undergo dynamic changes in gene expression and protein composition to adapt to changes in the local environment. When such changes are long-lasting, they are described as being due to “cellular plasticity.” The set of proteins expressed is determined by multiple parallel signal

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transduction pathways that ultimately regulate one or more events in transcription, translation, mRNA half-life and protein degradation. Transcriptional controls have been studied extensively in vascular cells, but epigenetic mechanisms contributing to smooth muscle phenotype are not as well defined. Dynamic changes in methylation of CpG sites in key promoters, histone modifications and microRNA-induced gene silencing are subjects of intense study in cardiovascular physiology. These phenomena are not nearly as well defined in smooth muscle cells as they are in cardiac muscle cancer cells and the immune system.[1-3]

The goal of this review is to summarize emerging knowledge of the microRNA (miRNA) class of small, noncoding RNAs in vascular smooth muscle cell phenotypes in normal tissue and in pulmonary diseases. We will focus on miRNAs with validated targets that are relevant to smooth muscle contractility and vascular development. The reader interested in miRNAs in endothelial cells and stem cells is referred to several excellent recent reviews of the subject and a recent review of miRNAs in pulmonary hypertension.[4-8] Our narrow approach to the topic in this review is justified by the compelling need to identify novel, druggable targets for modifying vascular remodeling. There is certainly continuing need to define sets of miRNAs that control both conserved (proliferation and cell survival) and unique (smooth muscle contractile protein expression) processes in all vascular cells and all cells in the lung. There is also an appealing opportunity to capitalize on current knowledge of miRNA-induced gene silencing in developing novel therapeutic approaches to pulmonary hypertension. To that end, we describe examples of RNAi-based therapy of animal models of cardiovascular and respiratory diseases. These studies provide an exciting proof of principle for RNAi therapy of lung diseases including pulmonary hypertension.

**Smooth muscle cell phenotypes**

Smooth muscle cells in vitro are highly plastic cells that are easily manipulated by altering culture conditions to favor a more contractile phenotype or a proliferative, secretory and migrating phenotype. Contractile characteristics are promoted by culturing at high density and in reduced serum concentrations in the presence of soluble factors including insulin, retinoic acid, and transforming growth factor beta 1 (TGF-β1). Some soluble factors are clearly downregulated by conditions promoting proliferation. In culture proliferation, cell migration and secretion of mediators of inflammation can be induced by serum-containing medium with the trophic growth factors epidermal growth factor and fibroblast growth factor. The gene expression profile of proliferative and migratory vascular smooth muscle cells is not as well defined as the contractile phenotype. It is frequently used in the context of cultured cells that proliferate in serum-containing medium, express a chemotactic response to platelet derived growth factor (PDGF), and secrete a variety of proteins. Secreted proteins include type I collagen, cytokines, chemokines and growth factors. Growth, migration and proliferation in vitro are thought to recapitulate organogenesis during fetal and neonatal development.

There are differing views of the two “phenotypes” of vascular smooth muscle. One view holds that switching from proliferating/migrating cells to contractile cells is a stable, mutually exclusive condition—a binary phenomenon.[11] An alternate view is that the phenotype of smooth muscle cells is graded with cells in a tissue having a mosaic pattern of contractile protein gene expression.[10,12,13] In either case, gene expression programs in smooth muscle appear to be highly adaptable depending on tissue type, culture conditions and disease processes.

Current progress in epigenetic mechanisms controlling gene expression strongly suggests part of the adaptability of vascular smooth muscle and other vascular wall cells is due to dynamic changes in gene expression. One topic of great interest is the influence miRNAs might have on networks of target genes that are important in disease progression. Defining the miRNAs and their targets in pulmonary vascular smooth muscle phenotype switching should add novel therapeutic targets for anti-remodeling drugs. Progress on this topic will have high impact on translational research aimed at developing novel treatments of pulmonary hypertension.

**MiRNA biogenesis and miRNA-induced silencing**

MiRNA biogenesis and the mechanisms of miRNA-induced gene silencing have been well described, and the basic steps appear to be highly conserved among various cell types. The current consensus on biogenesis is illustrated in Figure 1, and the interested reader is directed to recent reviews of the topic for more detailed description of the process.[14,15] Many miRNA genes are hosted within other genes distributed throughout mammalian genomes. They are often located in introns and sometimes in exons, and some are in intergenic
Transcription of primary micro RNA (Pri-miRNA) from miRNA genes is followed by cleavage to precursor mRNA (Pre-miRNA) by the Drosha nuclear RNase III. The Pre-miRNA is then exported to the cytoplasm by exportin via nuclear pore. In the cytoplasm, Pre-miRNA is further processed by RNase activity of Dicer to the mature micro RNA duplex. The duplex loads onto Argonaut ribonucleases in the RISC complex and separates. One of the mature miRNA strands (red strand) mediates small interfering RNA silencing by degrading the target mRNA or interfering with translation. The outcome of RISC formation varies with the degree of complementarity of the seed sequence of miRNA and 3’ untranslated regions (UTR) of the target mRNA.

Gene silencing mediated by the mature miRNAs then occurs by two somewhat different mechanisms that both require the mature miRNA to complex with several proteins including Argonaut family members Ago-1 and Ago-2. The mature dsRNA duplex loads into RNA-induced silencing complexes (RISCs) that mediate posttranscriptional silencing by reducing mRNA stability or by translational block depending on the degree of complementarity of the miRNA seed sequence (nucleotides 2-8) with the target sequence (Fig. 1). MiRNA tends to be cleaved by Ago-2 when complementarity is perfect, although this is not universally true. The transcript can then be further modified by uridylation and decapping and then completely degraded by exonuclease cleavage. When complementarity is imperfect a variety of miRNA/mRNA/RISC structures can form that block initiation, cause premature termination, and induce dissociation of ribosomes. MiRNA degradation then occurs following deadenylation, decapping and exonuclease action. Translation of sets of target proteins is thereby reduced.

For the purposes of this review we are interested in miRNA-mediated gene silencing, but the reader should be aware that instances of miRNA-mediated translation enhancement have also been reported.[20] The remainder of the review focuses on particular miRNAs that target genes important in vascular smooth muscle development, contraction and lifespan. The aim is to show how miRNA-induced silencing could alter smooth muscle progenitor differentiation, smooth muscle restricted contractile protein expression, smooth muscle proliferation, and proinflammatory mediator synthesis. Each of these processes participate in vessel wall remodeling that contributes to pathogenesis of pulmonary vascular diseases.

**MiRNAs and vascular smooth muscle plasticity**

The role of miRNA-mediated gene silencing in vascular smooth muscles was first described in 2007. The number of studies describing miRNAs expressed vascular tissues under a variety of conditions is growing exponentially. There are now reports of miRNAs necessary for normal vascular development as well as miRNAs that are altered in vascular diseases including vascular damage, atherosclerosis and pulmonary hypertension. These miRNAs are sorted into functional groups in Table 1 to illustrate the miRNAs known to contribute to smooth muscle cell fate.

The earliest reports linking miRNAs and vascular smooth muscle remodeling were studies of miRNAs upregulated during injury. Zhang and coworkers discovered miR-21 levels increased following carotid artery injury.[21] They then went on to establish that miR-21 promotes vascular smooth muscle proliferation by silencing expression of phosphatase and tensin homolog (PTEN) and increasing
expression of B-cell leukemia/lymphoma 2 (Bcl2). Paradoxically, miR-21 was subsequently shown by Davis et al.\textsuperscript{[19]} to promote contractile protein expression induced by TGF-β family proteins in cultured pulmonary artery vascular smooth muscle by silencing programmed cell death 4 (PDCD4). TGF-β family proteins enhanced processing of the miR-21 primary transcript to the mature miRNA, and increased miR-21 was found to enhance smooth-muscle restricted contractile protein expression.\textsuperscript{[19]} These findings are important because they were the first example of growth-factor regulation of miRNA processing in smooth muscle, and they showed that one miRNA (miR-21) under different conditions promotes either contractile or proliferative phenotypes. It remains to be seen whether other miRNAs also exert dual effects on smooth muscle phenotype, but the early studies point to the complexity and potential duplicity of miRNA targets and physiological effects.

The initial studies of miR-21 in vascular remodeling were quickly followed by a series of landmark studies of the miR-143–145 cluster. Neointimal lesion formation is associated with downregulation of miR-145 as well as downregulation of contractile protein expression and increased proliferation of neointimal cells.\textsuperscript{[22]} Downregulation of miR-143–miR-145 and downregulation of contractile protein expression was then shown to be cause-and-effect in a series of loss of function studies by Cordes et al.\textsuperscript{[23]} Studies in cultured vascular smooth muscle cells and knockout mice have defined a pathway for reciprocal control of Kruppel-like factor 4 (KLF4) and myocardin expression by miR-145 as shown in Figure 2. Knockout mouse studies have corroborated the initial cell culture studies and have verified that the miR-143–145 cluster is a dominant regulator of smooth muscle differentiation. The miR143–145 cluster enhances contractile protein expression required for contractility and proper blood pressure regulation.\textsuperscript{[24,26]} It also has a profound effect: to promote differentiation of stem cells to smooth muscle cells.\textsuperscript{[4]} MiR-145 can directly silence expression of KLF4 and can indirectly upregulate myocardin expression (Fig. 2), which contributes to TGF-β1 enhancement of serum response factor (SRF)-dependent contractile protein expression.\textsuperscript{[27]} SRF regulates a loosely coordinated set of smooth muscle contractile, cytoskeletal and matrix protein genes with CArG boxes in the 5' untranslated region.\textsuperscript{[24,28,29]}

In addition to regulating contractile protein expression, miR-143–145 also influences expression of proteins involved in matrix remodeling and cell migration. Downregulation of miR-143–145 upregulates formation of podosomes and upregulates expression of PDGF receptor, protein kinase C (PKC) epsilon and the actin bundling protein fascin.\textsuperscript{[30]} Podosomes are discrete sites of matrix remodeling necessary for invasive migration of vascular smooth muscle cells during vascular wall remodeling. Whether podosome formation is necessary for development of arteriopathy in pulmonary vascular diseases is unknown, but seems plausible given the extensive structural remodeling that

### Table 1: MicroRNAs regulating smooth muscle cell fate

| miRNA regulating SMC phenotype | miRNA regulating Synthetic functions | miRNA regulating Differentiation |
|--------------------------------|-------------------------------------|--------------------------------|
| miR-1\textsuperscript{[31]}    | miR-24\textsuperscript{[36]}         | miR-10a\textsuperscript{[38]}    |
| miR-25\textsuperscript{[32]}   | miR-25\textsuperscript{[32]}         | miR-143–145\textsuperscript{[4,7,59]} |
| miR-133a\textsuperscript{[60]} | miR-26a\textsuperscript{[61]}        | miR-155\textsuperscript{[62,63]} |
| miR-143–145\textsuperscript{[23,26]} |

### miRNA regulating SMC proliferation, migration and survival

| miRNA regulating Proliferation | miRNA regulating Apoptosis/Survival | miRNA regulating Migration/Cytoskeletal proteins |
|-------------------------------|-----------------------------------|-----------------------------------------------|
| miR-1\textsuperscript{[64]}   | miR-146a\textsuperscript{[33]}    | miR-21\textsuperscript{[21]}                |
| miR-21\textsuperscript{[21]}  | miR-204\textsuperscript{[44]}     | miR-143–145\textsuperscript{[24]}          |
| miR-26a\textsuperscript{[65]} | miR-221\textsuperscript{[21,37]}  |                                               |

Figure 2: MicroRNAs regulating smooth muscle restricted contractile protein expression. Multiple miRNAs modulate the key transcriptional co-regulators myocardin and KLF4, which are positive and negative regulators of SRF-dependent smooth muscle gene expression. Current evidence shows miR-1, miR-25, miR-133a, miR-146a and miR-145 all modulate expression of either KLF4 or myocardin to influence contractile protein expression. The red lines indicate silencing of protein expression or inhibition of miRNA expression by pathway components. The green arrows indicate activation or upregulation of the pathway component.
occurs in humans and in animal models of pulmonary hypertension.

In addition to the miR-143~145 cluster, other miRNAs can modulate smooth muscle gene expression by altering KLF4 expression (Fig. 2). KLF4 is a direct target of miR-1 in stem cell differentiation.[31] miR-25 in airway smooth muscle,[32] and miR-146a in vascular smooth muscle.[33] Regulation of KLF4 by miR-146a involves a feedback loop in which miR-146a silences KLF4 which competes with KLF5 to reduce transcription of the miR-146a gene. Neointima formation is thereby enhanced by smooth muscle cell proliferation and migration due in part to increased KLF4 expression. The proximal signals that activate the miR-146a-KLF4/KLF5 pathway are not defined in vascular smooth muscle. However, in airway smooth muscle expression of primary-miR-146a expression is activated by nuclear factor kappa beta signaling and primary-miR-146a processing is regulated by MEK-1/2 and JNK-1/2.[34] Mature miR-146a is also induced by stretch in C2C12 myoblast cells.[35] Defining the trigger and upstream transduction pathways in vascular smooth muscle might identify high-value targets for anti-remodeling therapy in PAH.

The peptide growth factors PDGF and TGF-β1 are known to regulate smooth muscle phenotype and to mediate vascular development and remodeling in PAH. PDGF-BB promotes the proliferative/migratory/secretory phenotype in culture and is necessary for proper formation of new blood vessels during development. In contrast, TGF-β family proteins often enhance the formation of new blood vessels during development. The PDGF signaling pathway in vascular smooth muscle also induces expression of miR-221 which might also contribute to neointimal proliferation.[37] MiR-221 is upregulated in a variety of cancers, and miR-221 silences expression of p27Kip1 during skeletal muscle differentiation.[38] In cultured human pulmonary artery smooth muscle, silencing p27Kip1 by miR-221 overexpression promotes proliferation.[39] In a separate study of rat aorta, smooth muscle miR-221 and miR-222 expression was induced with PDGF, which also decreased p27Kip1 and p57Kip2 expression.[39] MiR-221 and miR-222, like miR-21, are good examples of miRNAs that are conserved in many cells and have consistent effects on cell cycle control proteins in vascular smooth muscle cells. It will be important to determine whether modifying the highly conserved process of cell cycle transit with RNAi-based therapy can be an effective anti-remodeling strategy. The timing of such treatments during development of pulmonary vascular remodeling will be important as was suggested above for modifying growth factor signaling with miR-24 antagonists.

**MiRNAs and pulmonary hypertension**

Studies of miRNAs in vascular remodeling during development of pulmonary hypertension must consider the multifocal, multicellular nature of the changes in vascular structures. Vascular lesions involving multiple cell types are observed in humans and in animal models of pulmonary arterial hypertension.[40] It seems likely that phenotype modulation of endothelial cells, smooth muscle cells, fibroblasts and both resident and immigrating progenitor cell types occurs.[41] Therefore understanding miRNA expression patterns in each cell type as a function of disease development and degree of severity is vital for designing novel therapeutic strategies.

To this point we have focused on miRNAs in vascular smooth muscle cells; but there is also a significant literature on miRNAs in endothelial cells and various types of pluripotent cells that is highly relevant. Comprehensive discussion of this issue is beyond the scope of this review, but some key observations are worth making. A number of miRNAs described in smooth muscle cells (e.g., miR-21 and miR-221 in Figure 3) have conserved functions in differentiation, proliferation and survival of endothelial cells and other vascular mural cells.[46] The initial studies of miRNAs in the cardiovascular system cited above suggest some likely targets for RNAi-based antagonism of remodeling—e.g., miR-21, miR-145 and miR-221.[42] However, until recently it was not clear which miRNAs were altered during development of pulmonary arterial hypertension (PAH). MiRNAs that promote arterial muscularization, that increase cell survival or proliferation and promote endothelial to mesenchymal transitions are clearly of great interest.
miRNA expression can prevent progression of established PAH. Rapid advances are being made in RNAi therapy of several vascular diseases in which target miRNAs have been identified, some target proteins and processes have been identified, and some demonstration of effective drug delivery has been presented.

The study of miR-204 in PAH is unique because roles for miR-204 in myogenesis and in other vascular diseases have not been described before. MiR-204 may have a particular set of functions in the pulmonary circulation that differ qualitatively or quantitatively from its function in other vascular beds. It seems reasonable to speculate that miR-204 is one of several miRNAs that promote differentiation of vascular smooth muscle, and that downregulation of miR-204 might occur during pathogenic vascular remodeling in atherosclerosis and restenosis. However, there are no reports of a strong association or cause-effect in these other vascular diseases. Nevertheless, targets of miR-204 have been validated in other cell types. Some of the target proteins have important roles in smooth muscle cell physiology and vascular diseases: TGF-β receptor 2, epidermal growth factor (EGF) receptor signaling, forkhead box C1 (FOXCl), and runt-related transcription factor 2 (Runx2). It is not clear whether these other targets of miR-204 are also contributing to arteriopathy in pulmonary hypertension, but these are target proteins worthy of further study.

**CONCLUSIONS AND FUTURE DIRECTIONS**

MiRNA expression surveys have yielded several candidate molecules that could contribute to disease development involving remodeling of smooth muscles. The results of unbiased expression surveys and hypothesis-driven biochemical and functional validation studies have provided important insights into disease mechanisms and potential targets of new treatments of vascular remodeling (Table 1). Some conserved miRNAs have been described in vascular smooth muscle tissues and cells in culture that are known from the cancer literature. MiRNAs controlling cell proliferation and cell survival (miR-21, miR-221 and miR-222) are altered in a variety of diseases. Some miRNAs appear to serve important roles of several vascular diseases in which target miRNAs have been identified, some target proteins and processes have been identified, and some demonstration of effective drug delivery has been presented.

In the animal models of PAH Courboulin et al. found miR-204 was also downregulated in humans with PAH and in rat models of PAH. Delivery of a miR-204 mimic to rat lungs reduced the severity of the disease, providing an exciting proof of principle for rescuing vascular smooth muscle phenotype in vivo with RNAi-based therapy. Courboulin et al. also showed downregulation of expression of miR-204 in mononuclear cells from in blood of PAH patients. This raises the possibility that changes in miRNAs in plasma and leukocytes might be useful biomarkers of PAH pathogenesis.

In the earliest published study of miRNAs in pulmonary hypertension, Caruso et al. surveyed miRNA expression in total lung extracts from two rat models (chronic hypoxia and monocrotaline model) of PAH. MiR-21 was downregulated in the monocrotaline model and in lung samples from humans with PAH. In the same study miR-451 was upregulated in both models but no differences were detected in lung samples from control and PAH human subjects. This initial report showed that there are disease-related changes in miRNAs associated with development of PAH in both animals and humans. A subsequent study by Courboulin et al. found miR-204 was also downregulated in humans with PAH and in rat models of PAH. Delivery of a miR-204 mimic to rat lungs reduced the severity of the disease, providing an exciting proof of principle for rescuing vascular smooth muscle phenotype in vivo with RNAi-based therapy. Courboulin et al. also showed downregulation of expression of miR-204 in mononuclear cells from in blood of PAH patients. This raises the possibility that changes in miRNAs in plasma and leukocytes might be useful biomarkers of PAH pathogenesis.

In the animal models of PAH Courboulin et al. identified potential targets for miR-204. They found Stat3 activation was increased upon attenuation of miR-204 expression and that miR-204 directly regulates SHP2 by targeting its 3’UTR. They developed the signaling model shown in Figure 3 where decreased miR-204 increases SHP2, which by activating Src increases Stat3 activation. Stat3 is hypothesized to promote smooth muscle proliferation and pulmonary vessel wall thickening. This landmark study provides solid proof of principle that “rescue” of low

**Figure 3:** Signal transduction pathways implicated in pathogenesis of vascular remodeling relevant to pulmonary hypertension. Panel A: miR-21 may increase vascular smooth muscle cell number by targeting proteins that regulate cell proliferation (PTEN) and apoptosis (Bcl2). Changes in miR-21 expression have been observed in lung tissues and in vascular smooth muscle in animal models of pulmonary hypertension. Panel B: miR221 promotes vascular smooth muscle cell proliferation by silencing the cell cycle inhibitor p27Kip1. Panel C: miR-204 downregulated in pulmonary hypertension in animals and in human leukocytes can indirectly promote cell proliferation. Derepression of SHP2 expression activates a Src/Stat3 cascade that promotes vascular smooth muscle proliferation.

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genes and yet in other settings act as tumor suppressors and regulators of pluripotency.

A number of important questions are raised by the recent surge in knowledge about miRNAs in vascular diseases. One issue is related to the general question of the significance of dynamic changes in epigenetic mechanisms of gene expression in pulmonary vascular diseases. Do pulmonary vascular smooth muscle cells respond to environmental inputs by altering epigenetic factors such as DNA methylation patterns in CpG regions of promoters? Are there diagnostic or prognostic modifications of histones that prime particular genes for expression that is subsequently modulated by the miR-143~145 cluster? Will new assays of miRNA expression and miRNA processing during various stages of disease development illuminate some new biomarkers or new candidates for inhibiting pathological vascular remodeling? Can RNA mimics and antagonists be effective anti-remodeling drugs in vivo?

There are reasons for optimism that RNAi therapy might be a useful anti-remodeling approach. One of the earliest examples of RNAi “therapy” in animals was intranasal delivery of antisense oligonucleotides against a respiratory syncytial virus protein to the lungs of mice infected with the virus.\(^{[52,53]}\) RNAi therapy can also be scaled up for use in primates. A locked nucleic acid modified miR-122 when administered IV to green monkeys inhibits cholesterol synthesis.\(^{[54]}\) Recently RNAi therapies targeting smooth muscle remodeling have also been shown to be effective in animal models. Pulmonary hypertension and asthma in animal models are both responsive to lung-restricted delivery of RNAi drugs that rescue (miR-204),\(^{[44]}\) or antagonize (miR-145)\(^{[55]}\) miRNAs altered by the disease. There is also hope that atherosclerotic plaque stability might be susceptible to manipulation via systemic delivery of RNAi-based drugs.\(^{[46,54,57]}\) To address the question of effective RNAi-based anti-remodeling therapy, novel RNAi-based drugs and novel delivery methods must be developed in animal models and then rapidly moved to first-in-human trials. It is now clear that chemically stabilized antisense ribonucleotides and modified miRNAs can be effective “therapy” in animal models of vascular and respiratory diseases. With ongoing development in RNAi drug design and delivery, these approaches should soon be applied to humans.

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