MicroRNA-21 Integrates Pathogenic Signaling to Control Pulmonary Hypertension

Results of a Network Bioinformatics Approach

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Background—Pulmonary hypertension (PH) is driven by diverse pathogenic etiologies. Owing to their pleiotropic actions, microRNA molecules are potential candidates for coordinated regulation of these disease stimuli.

Methods and Results—Using a network biology approach, we identify microRNA associated with multiple pathogenic pathways central to PH. Specifically, microRNA-21 (miR-21) is predicted as a PH-modifying microRNA, regulating targets integral to bone morphogenetic protein (BMP) and Rho/Rho-kinase signaling as well as functional pathways associated with hypoxia, inflammation, and genetic haploinsufficiency of BMP receptor type 2. To validate these predictions, we have found that hypoxia and BMP receptor type 2 signaling independently upregulate miR-21 in cultured pulmonary arterial endothelial cells. In a reciprocal feedback loop, miR-21 downregulates BMP receptor type 2 expression. Furthermore, miR-21 directly represses RhoB expression and Rho-kinase activity, inducing molecular changes consistent with decreased angiogenesis and vasodilation. In vivo, miR-21 is upregulated in pulmonary tissue from several rodent models of PH and in humans with PH. On induction of disease in *miR-21*–null mice, RhoB expression and Rho-kinase activity are increased, accompanied by exaggerated manifestations of PH.

Conclusions—A network-based bioinformatic approach coupled with confirmatory in vivo data delineates a central regulatory role for miR-21 in PH. Furthermore, this study highlights the unique utility of network biology for identifying disease-modifying microRNA in PH. **(*Circulation*. 2012;125:1520-1532.)**

Key Words: microRNA ■ molecular biology ■ network biology ■ pulmonary heart disease ■ vasculature

Pulmonary hypertension (PH) is a complex vascular disease that is clinically defined as a maladaptive increase in pulmonary arterial pressure. Etiologies are varied and are classified in 5 subcategories.[1] Later stages of disease are dominated by a dysregulated balance of vascular effectors controlling vascular tone, cellular proliferation, and thrombosis. Currently, overarching pathogenic pathways, such as Rho-kinase signaling, are implicated as regulators of these effectors in PH.[2] Nevertheless, it remains unclear how such diverse upstream triggers of PH cause a common pathophenotype.

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Notably, transforming growth factor/bone morphogenetic protein (BMP) signaling, inflammatory signaling, and hypoxic stress represent distinct triggers of PH that are active across etiologies (as reviewed in References 1 and 2). The underpinnings of cross talk between these pathways in the pulmonary vasculature are only beginning to be understood.[3,4] Identification of integrating factors that functionally

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link these pathways to PH may be especially amenable to in silico predictive approaches.

Recently, microRNA molecules (miRNA), which are conserved, non–protein-coding RNA molecules, have been identified as essential mediators of a variety of genes and cellular processes. Their expression can be regulated in a transcriptional or posttranscriptional fashion. Inside the cell, miRNA negatively regulate gene expression by primarily binding to the 3′ untranslated regions of messenger RNA (mRNA) transcripts to repress translation and/or degrade mRNA. Efficient binding relies on Watson-Crick base pairing between the 7-nucleotide seed sequence of a given miRNA and its mRNA target, and several algorithms have accordingly been developed to predict mRNA targets of each miRNA.[5]

Owing to their pleiotropic vascular functions,[6] miRNA may coordinately regulate multiple disease pathways in the pulmonary vasculature, but their importance in PH is just beginning to emerge.[7] Initial attempts to identify miRNA involved in complex diseases such as PH by using existing predictive algorithms have been reported but remain unproven.[8,9] In the present study, we have used a network-based bioinformatics approach to identify miRNA that regulate multiple interacting targets in the same functional network to generate robust actions in PH in vivo. This concept has been proposed previously,[10] but it has yet to be utilized as a method by which to rank miRNA with the highest likelihood of critically affecting disease. Encompassed in this set, microRNA-21 (miR-21) is linked to hypoxia, BMP signaling, and inflammatory signaling and thus is predicted to regulate downstream pathways relevant to PH. However, current data are sparse regarding the specific function of miR-21 in the pulmonary vasculature in vivo. Driven by these network-based predictions, we demonstrate the direct relationship of miR-21 to multiple pathogenic stimuli, its dynamic regulation in PH, and its critical role in the control of PH in vivo. As a result, this study offers a rational mechanism-based strategy for ascertaining the integrative role of miRNA in PH and perhaps other complex pathophenotypes.

Methods

Network-Based Analyses

Using Medline (PubMed) and the search term *pulmonary hypertension*, we compiled a list of 131 genes that are directly implicated in the development of PH. We refer to this group of PH-relevant genes as the PH module (Table I in the online-only Data Supplement). Analysis of interactions between PH-relevant genes (PH network) was performed with the use of consolidated databases cataloguing a variety of molecular interactions, referred to as the consolidated interactome. The TargetScan 5 (Conserved) algorithm was used for miRNA target prediction.[11] Further details regarding the methods of network-based bioinformatics, including hypergeometric analysis,[12] are in Methods in the online-only Data Supplement.

Cells, Animals, and Human Reagents

Primary human pulmonary arterial endothelial cells (HPAECs), *VHL flox/flox;Cre-ER* mice, *IL-6* transgenic mice, *BMPR2*-heterozygous (+/−) mice, Sprague-Dawley rats, and *mmu-miR-21*–null (−/−) mice are described in Methods in the online-only Data Supplement. The Harvard Center for Comparative Medicine approved the use of animals in these experiments. Formalin-fixed, paraffin-embedded human pulmonary arterial hypertension lung specimens were collected from unused and discarded surgical samples; nondiseased human lung specimens have been described previously.[13] The Partners Healthcare institutional review board and the New England Organ Bank approved the use of these human specimens.

Induction of PH in Mice

Eight-week-old *miR-21*–null (−/−) and *miR-21*–wild-type (+/+) littermate mice were injected with SU5416 (20 mg/kg; Sigma-Aldrich), followed by exposure to normobaric hypoxia (10% O_2; OxyCycler chamber, Biospherix Ltd, Redfield, NY) or normoxia (21% O_2) for 1 to 3 weeks, as described previously.[14]

Right Heart Catheterization and Physiological Measurements

Systemic blood pressure was determined in unanesthetized mice by tail-cuff plethysmography (Visitech Systems).[15] Repeated values (10–20) were averaged at each determination. Right heart catheterization and measurement of right ventricular systolic pressure (RVSP) were performed as described previously for mice[16] and rats.[17]

Statistical Analysis

Unless indicated otherwise, all numeric quantifications represent mean±SEM for ≥3 independent experiments, each performed in triplicate (n=number of independent experimental repetitions). Images are representative of experiments that have been repeated at least 3 times. Paired samples were compared by Student *t* test. Comparison of multiple samples was performed by 1-way ANOVA followed by Student-Newman-Keuls post hoc tests (and confirmed by Tukey post hoc tests) to calculate *P* values. Values of $P \leq 0.05$ are considered significant.

Additional Information

See Methods in the online-only Data Supplement for a detailed description of manipulation of miRNA and mRNA expression in cultured cells, F-actin labeling, measurement of protein expression, and tissue analyses.

Results

A Network Biology–Based Approach Predicts Disease-Modifying miRNA in PH

To identify potential disease-modifying miRNA in PH, a list was derived of regulatory factors that are strongly suspected to influence this disease (the PH module; Table I in the online-only Data Supplement). On the basis of a highly sensitive and specific *in silico* miRNA target prediction algorithm, TargetScan 5 (Conserved),[11] of the 153 conserved "groups" of miRNA defined by identical seed sequences, a great majority (129) are predicted to target at least 1 member of the PH module (Figure IIA in the online-only Data Supplement). Thus, simply cross-referencing known PH-relevant genes with miRNA target lists offers little insight into which miRNA exert the most powerful influence on disease-relevant pathways.

To specifically identify miRNA that may robustly regulate disease phenotype by targeting multiple related genes in functionally integrated pathways, network analysis was employed to determine the functional interconnectivity among the PH-relevant target genes. With the use of the consolidated interactome (see Methods), mapping of known interactions among genes in the PH module revealed a dense network (ie, the PH network; Figure I in the online-only Data Supplement). This network includes 115 genes (of the 131 genes in the PH module, 115 were found in the consolidated interactome) with 255 direct interconnections (edges) between them

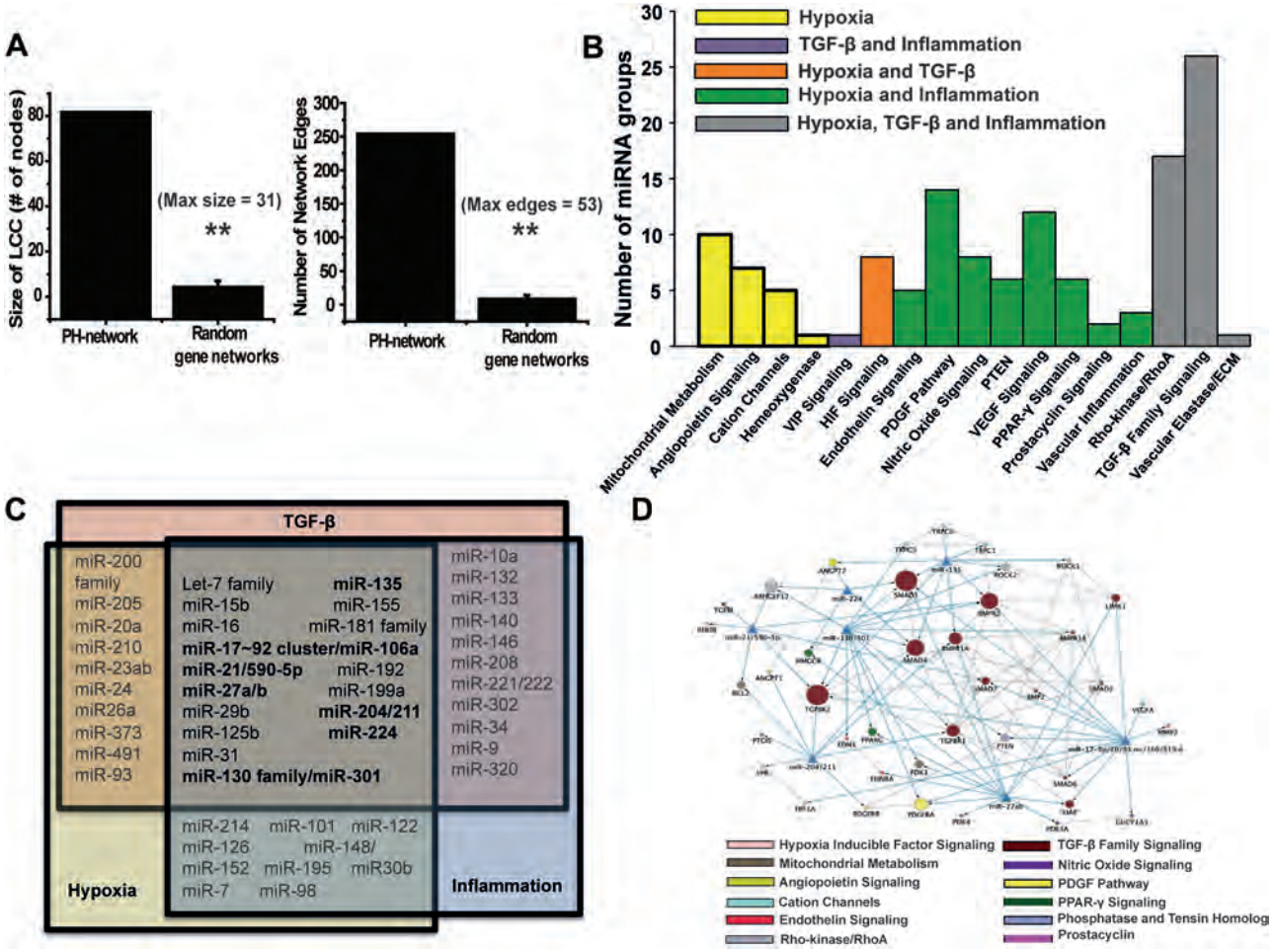

Figure 1. A network biology approach identifies pulmonary hypertension (PH)–modifying microRNA molecules (miRNA). **A**, The PH network displays substantial functional interconnections. Left, The mean largest connected component (LCC) size derived from 100 000 randomly chosen modules of 115 genes from the consolidated interactome (4.5±2.5, mean±SD) is significantly smaller than the LCC of the PH network (82 nodes). The maximum LCC size (max size) from randomly selected gene modules is 31. $^{**}P \ll 10^{-5}$). Right, The mean number of direct interconnections (edges) within 100 000 randomly chosen modules of 115 genes from the consolidated interactome (9.4±5.6, mean±SD) is significantly smaller than the number of edges in the PH network (255 edges). The maximum number of edges (max edges) within randomly selected gene modules is 53. $^{**}P \ll 10^{-5}$). **B**, MiRNA that associate with the PH network (29 miRNA groups) target a subset of pathways related to hypoxia, inflammation, and/or transforming growth factor (TGF)-β. **C**, A subset of miRNA previously associated with hypoxia, inflammation, and TGF-β is predicted to target the PH network. MiRNA groups predicted by enrichment analysis (Table 1) are bolded. **D**, Predicted target network of 7 miRNA groups identified both by enrichment analysis and literature review reveals genes that may represent points of coordinated miRNA regulation in PH. Circles indicate predicted gene targets; triangles, miRNA; blue lines, predicted associations of miRNA and targets; and dotted gray lines, gene interactions documented in the consolidated interactome. Circle size is proportional to the number of miRNA groups (among these 7) predicted to target that particular gene. VIP indicates vasoactive intestinal peptide; HIF, hypoxia-inducible factor; PDGF, platelet-derived growth factor; PPAR, peroxisome proliferator-activated receptor; PTEN, phosphatase and tensin homolog; and ECM, extracellular matrix.

and a largest connected component size of 82 nodes. Notably, both of these parameters are substantially larger than those generated from random gene associations (Figure 1A; left, largest connected component; right, edges). Thus, the size and dense interconnections of the PH network reflect its tendency to act in a functionally coordinated fashion, creating an ideal substrate with which to identify miRNA that preferentially target functionally related genes.

Having established the specific interconnectedness of the PH network, a hypergeometric analysis was then used to rank miRNA according to the proportion of their targets that interact with the PH network while adjusting for the variation in the total targets predicted for each miRNA within the entire consolidated interactome.[12] The use of the hypergeometric analysis allows for ranking of miRNA by the proportion of their predicted targets within the interconnected network, thus increasing the likelihood that a predicted miRNA will target functional networks of PH-relevant genes. This enrichment analysis identified 29 miRNA groups for which there exists a 5% or lower probability ($P \leq 0.05$) that the overlap of their predicted target list with the PH network occurred by chance (Table 1). We chose to further analyze only these miRNA because they represent the most likely candidates that regulate multiple genes and pathways to coordinate pathogenic effects within the PH network. Importantly, when the same hypergeometric analysis was performed on a group of poorly connected genes of the same size (eg, an alternative module of 115 randomly sampled genes with a largest connected component of 4 and 8 edges), no miRNA were

Table 1. A Subset of MicroRNA Predicted to Target the PH Network

MicroRNA	MiRNA Targets in PH Network	MiRNA Targets Outside PH Network	PH Network Genes That Are Not Targets	Remaining Genes in Consolidated Interactome	*P*
miR-130/301	14	502	101	11 026	0.0004
miR-21/590-5p	7	150	108	11 378	0.0008
miR-361/361-5p	5	91	110	11 437	0.0022
miR-135	10	357	105	11 171	0.0024
miR-375	5	102	110	11 426	0.0035
miR-204/211	9	320	106	11 208	0.0037
miR-873	5	108	110	11 420	0.0043
miR-17-5p/20/93.mr/106/519.d	14	671	101	10 857	0.0045
miR-216/216a	5	111	110	11 417	0.0048
miR-27ab	13	622	102	10 906	0.0059
miR-200bc/429	12	569	103	10 959	0.0074
miR-149	6	182	109	11 346	0.0082
miR-205	6	200	109	11 328	0.0120
miR-455/455-5p	4	94	111	11 434	0.0132
miR-224	5	161	110	11 367	0.0183
miR-153	8	370	107	11 158	0.0211
miR-145	8	372	107	11 156	0.0216
miR-410	7	303	108	11 225	0.0222
miR-383	3	63	112	11 465	0.0233
miR-148/152	8	381	107	11 147	0.0239
miR-219/219-5p	5	190	110	11 338	0.0312
miR-182	10	580	105	10 948	0.0351
miR-1/206	8	419	107	11 109	0.0352
miR-340/340-5p	11	672	104	10 856	0.0372
miR-290-5p/292-5p/371-5p	4	139	111	11 389	0.0395
miR-96/1271	9	527	106	11 001	0.0440
miR-24	6	288	109	11 240	0.0446
miR-190	3	85	112	11 443	0.0452
miR-221/222	5	217	110	11 311	0.0462

MicroRNA molecules (miRNA) are ranked by *P* value (with the lowest value indicating the least likelihood that the targets of a given miRNA are encompassed in the pulmonary hypertension [PH] network by random chance).

identified that recognize multiple targets and carry $P \leq 0.05$ (Figure IIB in the online-only Data Supplement). This finding reinforces the importance of the interconnectedness of the PH network in its ability to facilitate predictions of miRNA with functional significance in PH.

Notably, the PH-relevant targets of these 29 miRNA groups encompass pathways (Table II in the online-only Data Supplement) that are associated, by varying degrees, with hypoxia, inflammation, and transforming growth factor/BMP signaling (Figure 1B). When compared with a list of miRNA previously implicated in these signaling categories (Table III in the online-only Data Supplement), there is significant overlap. This latter list identifies >100 miRNA, only 17 of which relate to all 3 functional categories (Table III in the online-only Data Supplement and Figure 1C), a relatively high percentage of which (7 of 17 miRNA groups; Figure 1C, bolded miRNA) is also predicted by the hypergeometric enrichment analysis (Table 1). A map of the interactions in the PH network among the predicted targets of these 7 miRNA groups (Figure 1D) reveals a group of genes preferentially targeted by multiple miRNA that may be subject to coordinated regulation. Furthermore, included in these 7 miRNA groups is miR-204,[7] which has been implicated previously in PH pathogenesis, as well as the miR-17 to -92 family[18] and miR-21,[19,20] both of which have been associated previously with cellular phenotypes relevant to PH pathology. Thus, these multiple corroborating lines of evidence strongly indicate the importance of these 7 miRNA groups at the crucial intersection of hypoxia, inflammation, and transforming growth factor/BMP signaling in PH.

As a Predicted PH-Modifying miRNA, miR-21 Is Upregulated by Hypoxia and BMP Receptor Type 2 Signaling and Reciprocally Downregulates BMP Receptor Type 2 Expression

Driven by this network-based approach, miR-21 was chosen for validation as a central regulatory factor in PH given its high ranking and the availability of reagents to test this hypothesis. To

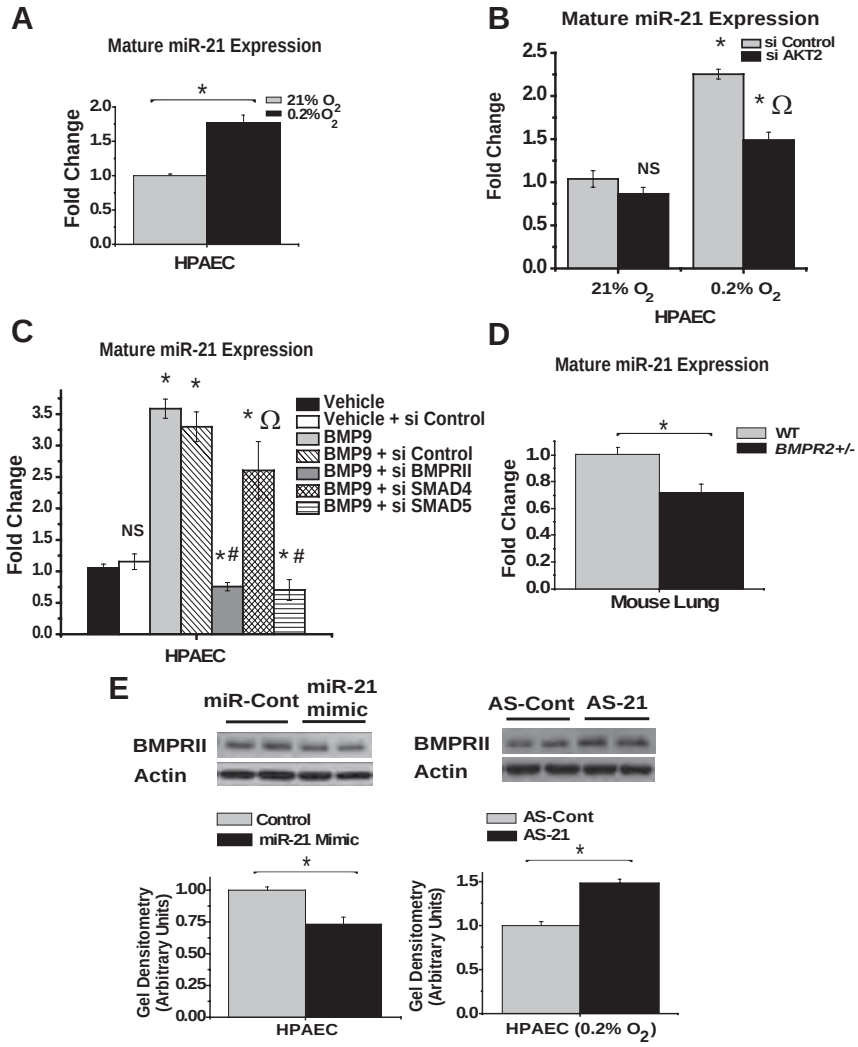

Figure 2. MicroRNA-21 (miR-21) is upregulated by hypoxia- and bone morphogenetic protein receptor type 2 (BMPRII)–dependent signaling and reciprocally downregulates BMPRII expression. **A**, As measured by reverse transcription polymerase chain reaction, hypoxic human pulmonary arterial endothelial cells (HPAECs) display increased levels of mature miR-21. **B**, Knockdown of AKT2 (si AKT2) negligibly affects miR-21 expression during normoxia but partially inhibits the specific upregulation of miR-21 during hypoxia. *$P \leq 0.05$ compared with si control samples at 21% O_2; $\Omega P \leq 0.05$ compared with si control samples at 0.2% O_2. **C**, Exposure to BMP9 increases mature miR-21 expression in HPAECs; BMP9-dependent upregulation of miR-21 is abrogated by knockdown of BMPR2 or SMAD5 but not SMAD4. *$P \leq 0.05$, NS signifies $P > 0.05$ compared with vehicle control; $\Omega P > 0.05$ compared with BMP9+si control; #$P \leq 0.05$ compared with vehicle+si control. **D**, Lungs of *BMPR2* (+/−) mice display reduced expression of miR-21 compared with wild-type littermates (WT). **E**, Left, As measured by immunoblot densitometry, BMPRII expression is decreased in HPAECs previously transfected with miR-21 oligonucleotide mimics. Right, BMPRII expression is increased in hypoxic HPAECs after inhibition of miR-21 (AS-21). In **A** through **D**, control miR-21 expression is assigned a fold change of 1, with which other conditions are compared. In **E**, immunoblots are representative of experiments performed at least in triplicate; gel densitometry is normalized to actin levels and compared as arbitrary units. In all panels, error bars reflect SEM; *$P \leq 0.05$ ($n \geq 3$), NS signifies $P > 0.05$ ($n \geq 3$). For all experiments, $n \geq 3$. AS indicates antisense.

confirm these predictions, the regulation of miR-21 by upstream triggers of PH was assessed in cultured HPAECs, which contribute substantially to dysregulated pathophenotypes seen in PH.[1]

In regard to hypoxia, expressions of mature miR-21 ($P=0.002$; Figure 2A) and its precursor, pri-miR-21 ($P=0.003$; Figure IIIA in the online-only Data Supplement), are increased by ≈2-fold in HPAECs after chronic exposure to 0.2% O_2, corroborating prior findings in transformed cells.[21] Moreover, during normoxia, forced expression of a master transcription factor of hypoxia, hypoxia-inducible factor (HIF)-1α, increases miR-21 expression ($P=0.03$; Figure IIIB in the online-only Data Supplement). However, because HIF-1α does not appear to bind the miR-21 promoter for direct transcriptional induction,[22] hypoxic upregulation of miR-21 may instead rely indirectly on HIF-dependent and/or HIF-independent factors. Recently, it was demonstrated that the activity of AKT2 is essential for the transcriptional upregulation of miR-21 in hypoxic transformed cells.[23] Correspondingly, in hypoxic HPAECs, inhibitory RNA knockdown of AKT2 (>80% transcript knockdown; Figure IVA in the online-only Data Supplement) partially represses the upregulation of miR-21, indicating its essential role in the control of this miRNA in hypoxic pulmonary vascular cell types ($P=0.03$; Figure 2B).

Similarly, we explored whether BMP receptor type 2 (BMPRII) specifically regulates miR-21. In HPAECs, BMP9 upregulates miR-21 by >3-fold ($P=0.008$; Figure 2C). To determine whether BMPRII and its downstream SMAD partners mediate this process, specific inhibitory RNAs were used that downregulate transcript expression of *BMPR2*, *SMAD4*, or *SMAD5* by at least 80% (Figure IVB through IVD in the online-only Data Supplement). Upregulation of miR-21 is abrogated by knockdown of BMPRII (Figure 2C). In correlation, lungs of mice that carry a heterozygous genetic deficiency in *BMPR2*[24] display a 30% decrease in expression of miR-21 ($P=0.03$; Figure 2D). Consistent with prior reports,[19,25] in HPAECs, knockdown of SMAD5, an intracellular receptor-SMAD factor that interacts with BMPRII, also inhibits upregulation of miR-21, but the same response is not observed after knockdown of SMAD4, a downstream partner of receptor-SMADs (Figure 2C). Thus, BMPRII is essential for the upregulation of miR-21 in response to BMP stimulation, and this process depends on certain, but not all, canonical SMAD factors.

In addition to these upstream processes, miR-21 can recognize a target sequence in the 3′ untranslated region of *BMPR2* transcripts,[26] but endogenous regulation has not been demonstrated. As assessed by immunoblotting, forced ex-

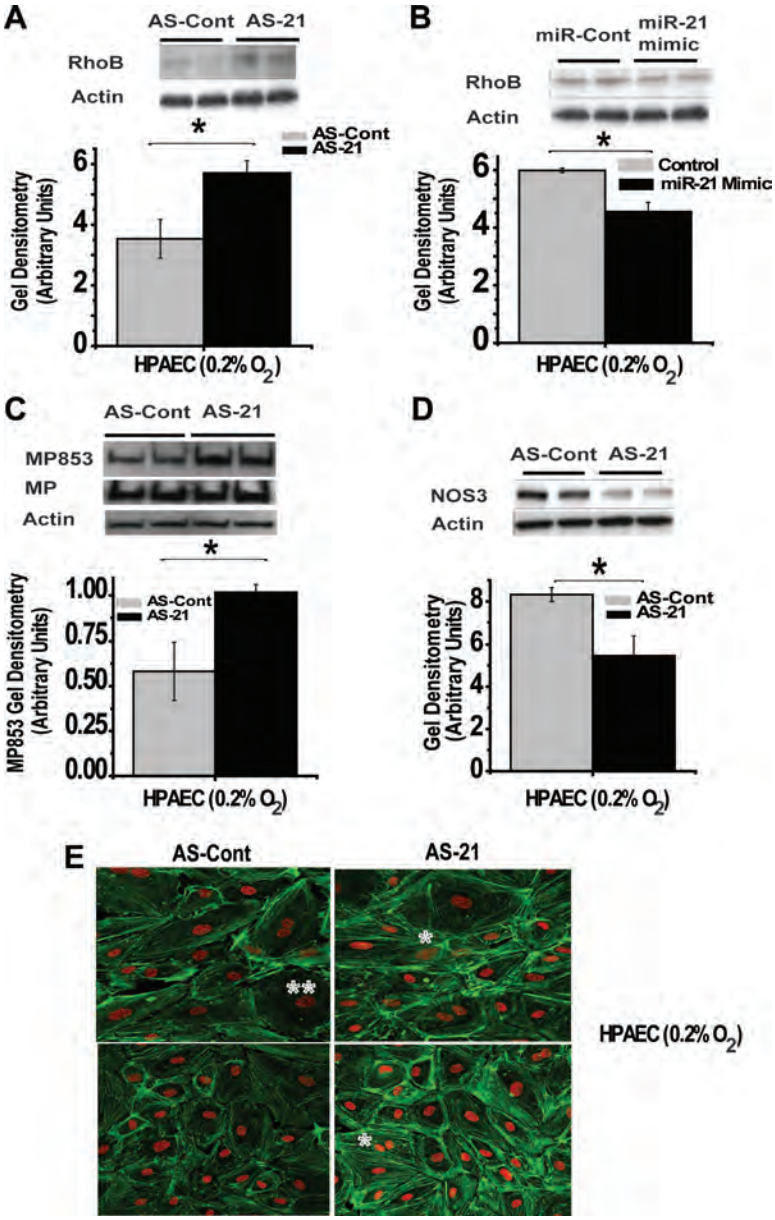

Figure 3. MicroRNA-21 (miR-21) inhibits RhoB expression to suppress Rho-kinase activity in human pulmonary arterial endothelial cells (HPAECs). **A** and **B**, As measured by immunoblot densitometry, RhoB expression is increased in hypoxic HPAECs after inhibition of miR-21 (**A**) and is decreased in hypoxic HPAECs transfected with miR-21 mimic (**B**). **C**, Rho-kinase–dependent phosphorylation of threonine-853 (MP853) in myosin phosphatase (MP) is increased in hypoxic HPAECs after inhibition of miR-21. **D**, Endothelial nitric oxide synthase (NOS3) expression is decreased in hypoxic HPAECs after inhibition of miR-21. **E**, Intensity of staining with phalloidin-FITC (green) to detect F-actin formation is increased in hypoxic HPAECs after inhibition of miR-21. Furthermore, as demonstrated by more fibers traversing nuclei, increased complexity of F-actin network formation is evident in AS-21–transfected cells (asterisk) compared with AS-Cont–transfected cells (double asterisk). In **A** through **D**, error bars reflect SEM; $*P \leq 0.05$ ($n \geq 3$), NS signifies $P > 0.05$ ($n \geq 3$). Immunoblots and micrographic images are representative of experiments performed at least in triplicate; gel densitometry is normalized to actin levels and compared as arbitrary units. For all experiments, $n \geq 3$. AS indicates antisense.

pression of miR-21 decreases BMPRII expression by ≈25% in HPAECs ($P=0.02$; Figure 2E, left), and inhibition of miR-21 increases BMPRII protein expression by 40% during hypoxia ($P=0.004$; Figure 2E, right) when endogenous miR-21 levels are augmented. Taken together with the upregulation of miR-21 by hypoxia and BMPRII activity, these data validate the predicted functional connection between miR-21 and 2 major pathogenic triggers of PH, hypoxia and BMPRII-dependent signaling.

MiR-21 Represses RhoB to Suppress Rho/Rho-Kinase Activity and Induce Molecular Changes Consistent With Decreased Angiogenesis and Vasodilation in HPAECs

Given this upstream control of miR-21 expression, the targets of miR-21 coinciding directly with the PH module (Table II in the online-only Data Supplement) were analyzed for their known regulation of vasoactive phenotypes. The small GTPase RhoB, a previously validated target of miR-21 in tumor cells,[27] emerged as a key candidate, given its function in activating Rho-kinase, which in turn can drive pulmonary vascular remodeling and PH in rodents and humans (as reviewed by Connolly and Aaronson[28]). Furthermore, among the several candidate PH-modifying miRNA, only miR-21 is predicted to regulate RhoB (Figure 1D). Thus, other miRNA are unlikely to compensate for changes in RhoB if miR-21 levels are altered in PH. In support of this hypothesis, in HPAECs, antisense inhibition of activated, endogenous miR-21 during hypoxia increases RhoB protein expression by nearly 60% ($P=0.02$; Figure 3A). Conversely, forced overexpression of miR-21 in hypoxic cultured HPAECs decreases RhoB protein expression by 30% ($P=0.01$; Figure 3B). Notably, this decrease is modest, perhaps reflecting the high baseline levels of endogenous miR-21 in HPAECs[29] and likely indicating that loss-of-function experiments in this context are more useful than forced expression to determine

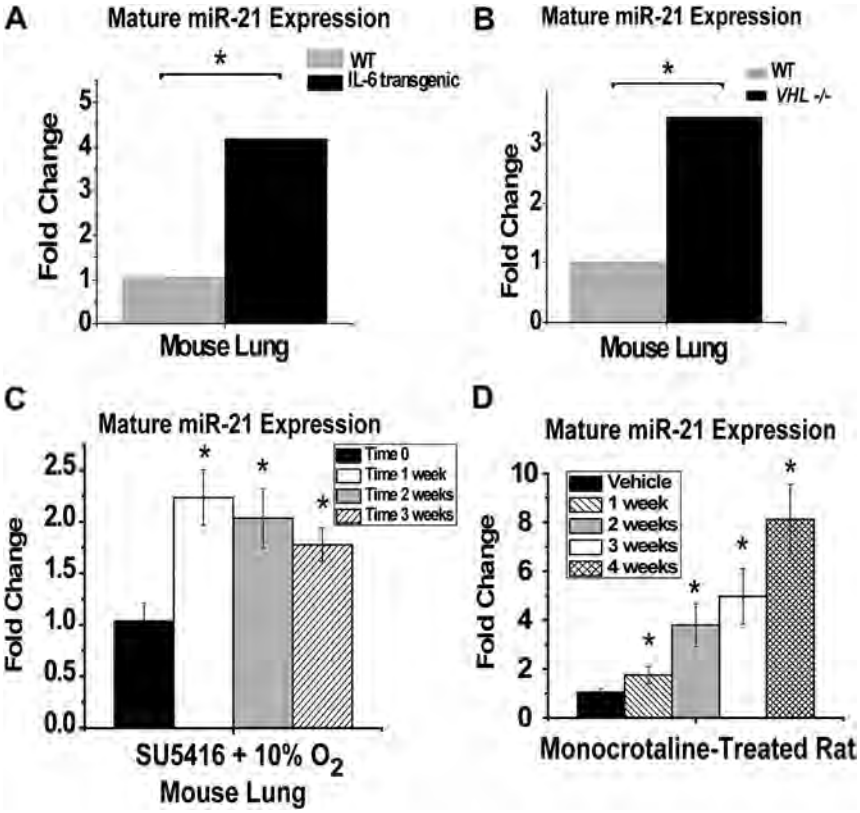

Figure 4. MicroRNA-21 (miR-21) expression is increased in in vivo models of pulmonary hypertension. **A**, MiR-21 expression is increased in mouse lung overexpressing interleukin-6 (IL-6) (n=5 mice per group). **B**, Reverse transcription polymerase chain reaction reveals that miR-21 is upregulated in the lungs of mice with deficiency of the Von Hippel Lindau protein *(VHL*-null) compared with wild-type (WT) littermates (n=6 mice per group). **C**, MiR-21 is upregulated in the lungs of mice exposed to a combination of SU5416 and 10% O_2 for 1 to 3 weeks compared with time 0 (n=3 mice per group). **D**, MiR-21 steadily increases over time in lungs of rats after 1 to 4 weeks after monocrotaline exposure (n=3 rats per treatment group) compared with time 0. Control miR-21 expression is assigned a fold change of 1, with which other conditions are compared. In all panels, error bars reflect SEM; *$P \leq 0.05$ (n≥3), NS signifies $P > 0.05$ (n≥3).

miR-21 function. Accordingly, in response to knockdown of miR-21, Rho-kinase activity increases, as demonstrated by a nearly 50% elevation of Rho-kinase–dependent phosphorylation of threonine-853 in myosin phosphatase[30] (Figure 3C). Thus, endogenous miR-21 directly represses RhoB and Rho-kinase activity in hypoxic HPAECs.

In response to elevated Rho activity, HPAECs typically display phenotypic responses that facilitate angiogenesis and vasoconstriction, which drive vascular remodeling and dysfunction in PH.[1] Among its several vasoconstrictive mechanisms, Rho-kinase activation represses endothelial nitric oxide synthase.[31] Correspondingly, inhibition of miR-21, which induces RhoB and Rho-kinase activity, decreases hypoxic endothelial nitric oxide synthase expression by nearly 40% (Figure 3D). Rho activation also induces formation of actin stress fibers (F-actin) to control angiogenesis.[32] Correspondingly, inhibition of miR-21 during hypoxia increases F-actin, as assessed by increased phalloidin-FITC immunofluorescence and increased complexity of the F-actin network (Figure 3E). As a result, reflecting its repressive actions on Rho/Rho-kinase activity, miR-21 induces molecular changes consistent with decreased angiogenesis and vasodilation in hypoxic HPAECs, which may counteract the pathogenic mechanisms critical to PH.[1]

MiR-21 Expression Is Increased in Remodeled Pulmonary Vessels of Animal Models of PH and Human PH

To validate these findings in vivo, regulation of miR-21 was assessed in distinct rodent models of PH as well as human lung obtained from PH patients. First, in lung tissue of transgenic mice overexpressing human interleukin-6 (IL-6), an inflammatory model of severe PH,[33] miR-21 is upregulated 4-fold ($P=0.03$; Figure 4A). Second, a hypoxia-induced model of PH was studied whereby conditional homozygous deficiency of the Von Hippel Lindau protein (*VHL*-null) leads to constitutive expression of master transcriptional regulators of hypoxia, HIF-1α and HIF-2α.[34] Similar to mice carrying the naturally occurring Chuvash loss-of-function *VHL* mutation,[35] *VHL*-null mice manifest increased RVSP, consistent with hypoxia-induced PH ($P<0.001$; Figure VA in the online-only Data Supplement). Correspondingly, miR-21 is upregulated 3-fold in lung tissue of *VHL*-null mice ($P=0.007$; Figure 4B). MiR-21 expression in whole lung homogenate of mice is not consistently altered after exposure to 10% oxygen for 3 weeks (Figure VB in the online-only Data Supplement). However, when chronic hypoxia is combined with administration of the vascular endothelial growth factor receptor-2 antagonist SU5416 in mice, a well-established set of exposures that can induce severe PH in rats[36] and mice,[37] an ≈2-fold increase (compared with time 0) in miR-21 is observed. This elevation persists over time ($P<0.03$ when comparing time 0 with all subsequent time points; Figure 4C) and correlates with increased RVSP and PH ($P<0.01$ when comparing time 0 with all subsequent time points; Figure VC, left, in the online-only Data Supplement). Finally, in the lung tissue of rats exposed to monocrotaline, a well-established rodent model of PH resulting from pulmonary endothelial injury and intense inflammatory reaction,[38] pulmonary expression of miR-21 increases over time (1–4 weeks after monocrotaline exposure) ($P<0.02$ when comparing time 0 with all subsequent time points; Figure 4D), which correlates with increased RVSP and PH ($P<0.005$ for time points 2–4 weeks after exposure compared with time 0; Figure VD in the online-only Data Supplement).

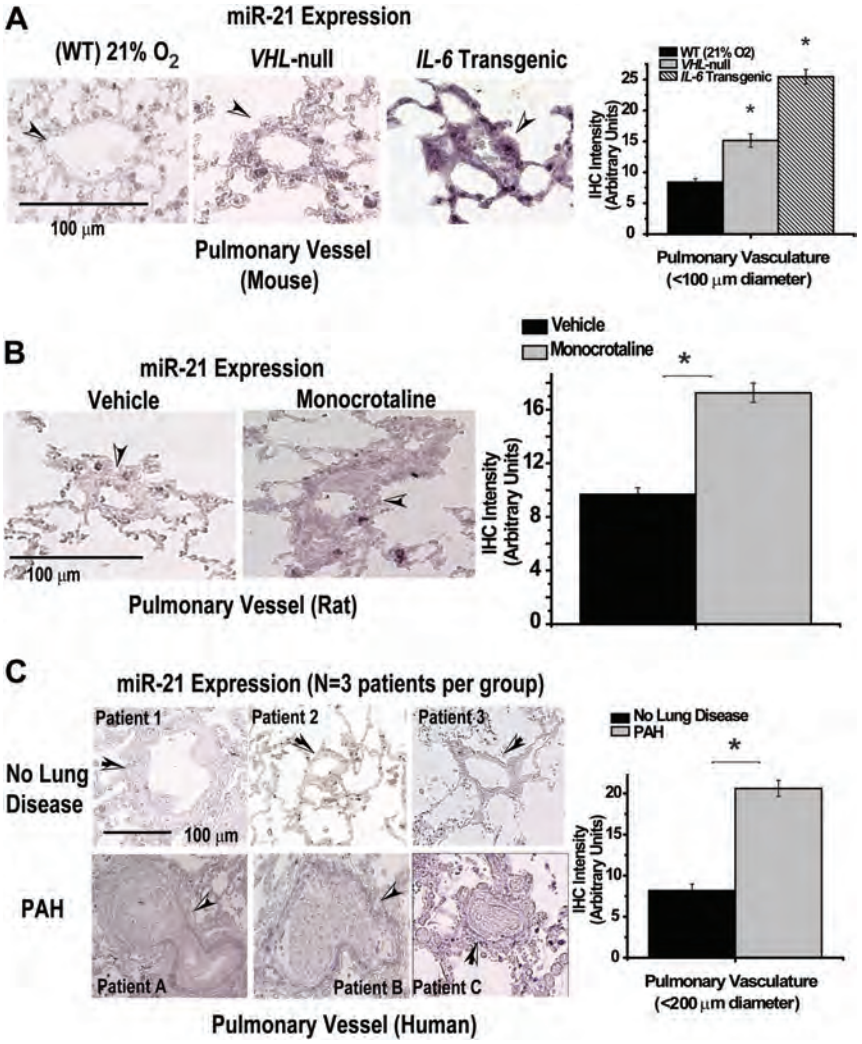

Figure 5. MicroRNA-21 (miR-21) expression is increased in remodeled pulmonary vessels in animal models of pulmonary hypertension and human patients with pulmonary arterial hypertension (PAH). **A** and **B**, In situ staining and quantification reveal increased expression of miR-21 in <100-μm pulmonary vessels of mice with deficiency of the Von Hippel Lindau protein *(VHL*-null) and interleukin-6 (*IL-6*) transgenic mice compared with wild-type (WT) control mice (n=3 mice per group) (**A**) and in <100-μm pulmonary vessels of monocrotaline-exposed rats compared with vehicle (n=3 rats per group) (**B**). IHC indicates immunohistochemistry. **C**, Increased expression of miR-21 in <200-μm pulmonary vessels from lungs of 3 human patients with PAH (patients A, B, C) compared with 3 nondiseased human lung specimens (patients 1, 2, 3). In all panels, error bars reflect SEM; *$P \leq 0.05$ (n≥3), NS signifies $P > 0.05$ (n≥3).

In correlation with previous reports of ubiquitous miR-21 expression,[39] in situ histological staining for miR-21 in mouse lung specimens reveals uniform but modest staining throughout the parenchyma (alveoli and small pulmonary vessels; Figure VE in the online-only Data Supplement). Importantly, corresponding with the reverse transcription polymerase chain reaction data from whole lung homogenate (Figure 4), increased in situ staining for miR-21 is particularly evident in the small (<100 μm) pulmonary vessels in *VHL*-null mice and *IL-6* transgenic mice ($P<0.02$ in both models; Figure 5A) as well as in monocrotaline-treated rat ($P<0.01$; Figure 5B). Increased staining for miR-21 is also evident in diseased pulmonary vessels (<200 μm) derived from 3 human patients (Table 2) with pulmonary arterial hypertension, a class of PH defined by characteristic histopathology and the absence of left heart failure, compared with 3 previously described nondiseased human lung samples[13] ($P<0.0001$; Figure 5C). Thus, increased pulmonary vascular expression of miR-21 is common to a variety of etiologies of PH in both rodents and humans.

Targeted Deletion of miR-21 Increases Rho-Kinase Activity and Alters the Development of PH In Vivo

To assess definitively the function of miR-21 in regulating the development of PH, disease severity was compared in mice genetically replete or deficient in miR-21 (+/+ or −/−). Construction and validation of an *miR-21*–null mouse

Table 2. Clinical Characteristics of Patients With Pulmonary Arterial Hypertension

Patient ID	Age, y	Gender	Mean Pulmonary Artery Pressure (Right Heart Catheterization), mm Hg	Right Ventricular Systolic Pressure (Echocardiography), mm Hg	Clinical Description
A	34	Female	50	107	Cardiopulmonary arrest (autopsy)
B	64	Female	55	106	Cardiopulmonary arrest (autopsy)
C	68	Female	44	83	Scleroderma

ID indicates identification.

have been described elsewhere.[40] At baseline, they are deficient in pulmonary expression of miR-21 (Figure VIA in the online-only Data Supplement). They demonstrate no differences from wild-type littermates in pulmonary arterial histology (Figure VIB in the online-only Data Supplement) or RVSP (Figure VIC in the online-only Data Supplement).

Given its specific induction of miR-21 in wild-type mice (Figure 4C), injury with SU5416 and chronic (3-week) exposure to normobaric 10% oxygen was employed to better discern a role for miR-21 in controlling PH. Compared with normoxia, both hypoxia ($P=0.02$) and hypoxia with SU5416 ($P=0.001$) induce similar increases in RVSP in wild-type mice, but these conditions do not demonstrate dramatic differences in RVSP when compared with each other ($P>0.05$; Figure VC, right, in the online-only Data Supplement). However, after exposure to SU5416 and chronic hypoxia, RVSP is significantly and consistently elevated in *miR-21*–null mice compared with wild-type littermate control mice ($P=0.0007$; Figure 6A). Such differences are not the result of varying effects on polycythemia or systemic blood pressure because hematocrit levels and systolic arterial blood pressures are similar in treated *miR-21*–null and wild-type mice (Figure VID and VIE in the online-only Data Supplement). Right ventricular remodeling is also evident in *miR-21*–null mice, as assessed by an increase in the mass ratio of the right ventricle to left ventricle and septum (RV/LV+S) ($P=0.01$; Figure 6B). Moreover, immunohistochemical staining of α-smooth muscle actin in the small pulmonary vessels ($\leq$100 μm diameter) of *miR-21*–null mice demonstrates exaggerated pulmonary vascular remodeling, as quantified by an increase in the overall ratio of muscularized vessel wall thickness to vessel diameter ($P<0.001$; Figure 6C). Consistent with the repression of RhoB and Rho-kinase activity by miR-21 in HPAECs (Figure 3), RhoB expression is elevated in *miR-21*–null mice in both the vascular intima and media of small pulmonary vessels ($<$100 μm in diameter) as assessed by immunohistochemistry ($P<0.001$; Figure 6D). Correspondingly, these vessels display increased Rho-kinase–dependent levels of phosphorylated myosin phosphatase in *miR-21*–null mice ($P=0.01$; Figure 6E). Furthermore, pulmonary tissue derived from treated *miR-21*–null mice exhibits a substantial increase in the transcriptional expression of at least 1 Rho-dependent vasoconstrictive effector of PH,[1] endothelin-1 ($P=0.02$) (Figure 6F). Thus, endogenous miR-21 is necessary for repression of both RhoB and Rho-kinase activity in the hypoxic pulmonary vasculature in vivo. In response, as demonstrated by these independent physiological and histological parameters, *miR-21*–null mice display exaggerated manifestations of PH, thus identifying a central regulatory role for miR-21 in this disease.

Discussion

In the present study, a unique network biology approach coupled with experimental data in cultured cells, animal models, and diseased human tissue identifies miR-21 as a critical regulatory factor that controls the downstream development of PH. This is the first report describing a reliable network-based bioinformatic approach to identify miRNA that control PH. As a result, we present a molecular model of the pulmonary vasculature whereby hypoxia, inflammation, and BMP-dependent signaling upregulate miR-21 (Figure 7). In response, miR-21 represses Rho-kinase activation, and perhaps other pathways, as a homeostatic brake to protect against PH in vivo.

The direct regulation of Rho/Rho-kinase by miR-21 augments our evolving understanding of this pathway in the diseased pulmonary vasculature. Upregulation of RhoA is known to induce Rho-kinase activity, leading to pulmonary vascular pathology.[28] Less is known about the functions of RhoB in the vasculature, although, in cell culture, it suppresses angiogenesis[41] and can induce a vasoconstrictive phenotype by activating endothelin-1[42] and repressing endothelial nitric oxide synthase.[31] Thus, our findings emphasize the underappreciated importance of RhoB in control of the dysregulated pulmonary vessel.

Furthermore, integration of various PH-relevant stimuli by miR-21 correlates with the known pleiotropic activity of this miRNA in various pathological contexts (as reviewed in Reference 39). Although modulation of RhoB reflects an important function of miR-21, other direct targets of miR-21 may affect downstream PH development. Among these, BMPRII may represent a key target given its role in PH development (as reviewed in Reference 1), as well as our finding that miR-21 regulates its expression (Figure 2E). However, unlike for RhoB, several PH-relevant miRNA are predicted to target BMPRII (Figure 1D), making the precise control of the BMP pathway by miR-21 more complex. Additional gene targets of miR-21 could influence PH, particularly tumor suppressor genes such as PDCD4.[19,20,39] However, PDCD4 is not substantially altered in *miR-21*–null mice with exaggerated PH (Figure VII in the online-only Data Supplement), consistent with a prior study of cardiac-specific PDCD4 in *miR-21*–null mice.[43] Thus, control of each direct target by miR-21 clearly differs depending on biological context. Accordingly, future comparison of the roles of miR-21 in different cellular and disease contexts may further highlight these differences.[1]

Beyond these specific insights regarding miR-21, additional PH-relevant miRNA that were identified by the network-based approach await validation. Of 4 other top-ranked miRNA (miR-20a, miR-27a, miR-130a, and miR-375; Table 1) that are expressed in the lung, directionally consistent changes in expression are observed for miR-20a, miR-27a, and miR-375 in 2 animal models of PH (accompanied by alteration of miR-130a in the hypoxia+SU5416 mouse model) (Figure VIIIA and VIIIB in the online-only Data Supplement). This supports the value of these predictions and, given the significant overlap of their targets (Figure 1D), emphasizes the need to consider coordinate control of PH progression by networks of miRNA and their integrated targets.

Although some of the same miRNA predictions are certainly possible through other methods besides network bioinformatics, such a network-based approach appears well suited to better predict the influence of miRNA on other complex diseases. On the basis of the weak predictive power of a poorly connected gene module (Figure IIB in the online-only Data Supplement), simple assembly of gene sets without

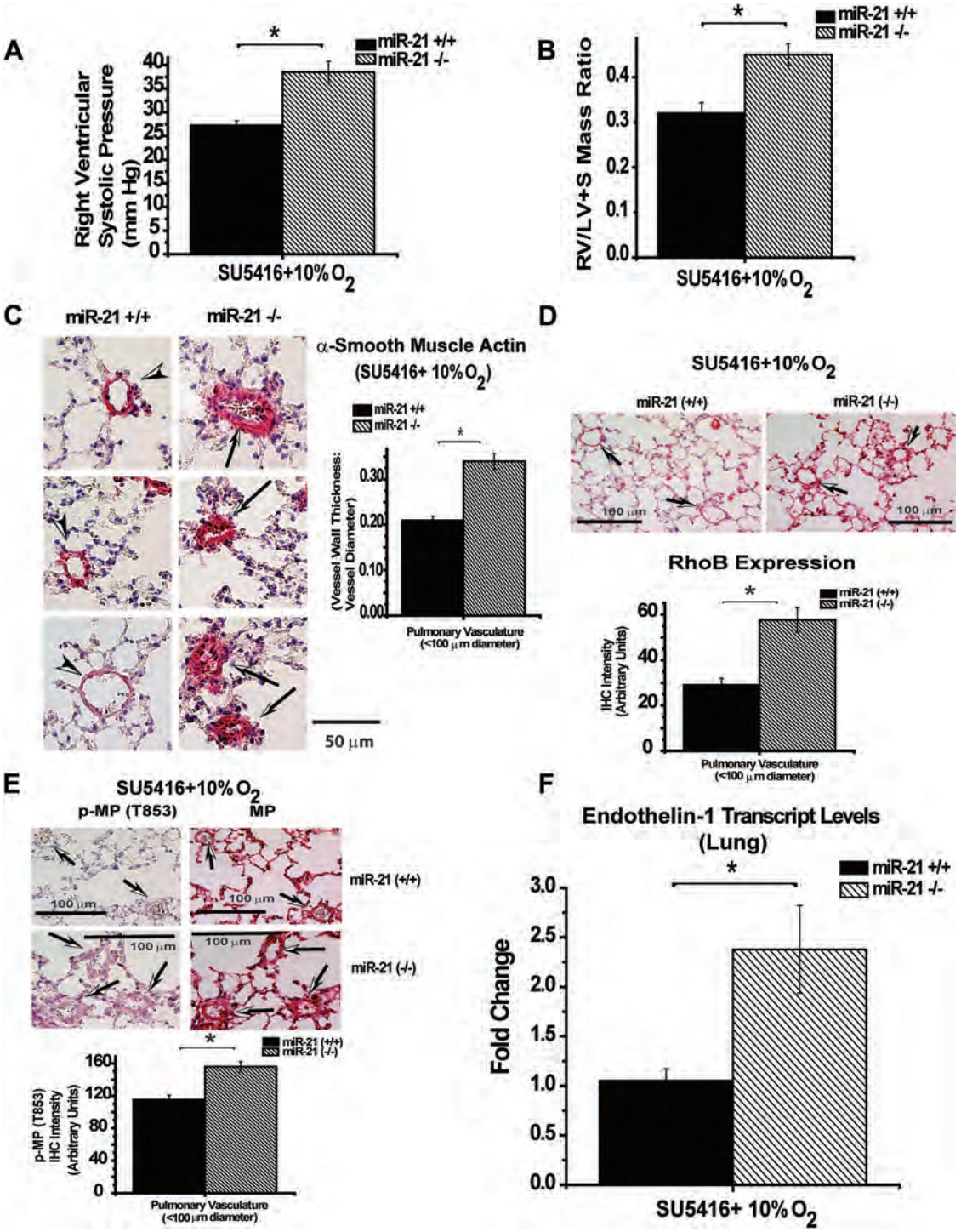

Figure 6. MicroRNA-21 (*miR-21*)–null (−/−) mice display exaggerated manifestations of pulmonary hypertension when exposed to SU5416 and chronic hypoxia. **A** through **C**, After exposure to SU5416 and 10% O_2, *miR-21*–null mice display increased right ventricular systolic pressure (**A**), increased relative right ventricular mass (mass ratio of right ventricle to left ventricle and septum [RV/LV+S]) (**B**), and increased pulmonary vascular remodeling (**C**). In **C**, remodeling is evident in *miR-21*–null mice (right, arrows), with increased medial thickening and cellularity, compared with pulmonary vessels (left, arrowheads) in wild-type littermates (α-smooth muscle actin stain). A significant increase in medial wall thickness relative to vessel diameter in <100-μm pulmonary vessels (right) is evident in *miR-21*–null mice (n=8 mice per treatment group in **A** through **C**). **D**, Immunohistochemistry (IHC) (arrows) and quantification (graph) reveal increased expression of RhoB in <100-μm pulmonary vessels of treated *miR-21*–null (−/−) mice (n=5 mice per group). **E**, Rho-kinase–dependent expression of phosphorylated myosin phosphatase [p-MP (T853)] (arrows, left) is increased in <100-μm pulmonary vessels of treated *miR-21*–null mice. Stain for total myosin phosphatase in consecutive sections is shown (MP) (arrows, right) (n=5 mice per group). **F**, Lung tissue from treated *miR-21*–null mice display increased transcript levels of endothelin-1. Wild-type expression is assigned a fold change of 1, to which expression in *miR-21*–null mice is compared (n=4 mice per group). In appropriate panels, error bars reflect SEM; *$P \leq 0.05$ ($n \geq 3$), NS signifies $P > 0.05$ ($n \geq 3$).

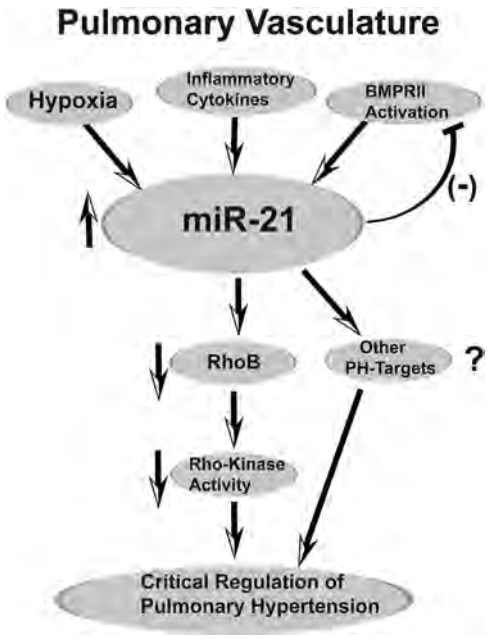

Figure 7. MicroRNA-21 (miR-21) integrates multiple pathogenic signals to regulate pulmonary hypertension (PH). A molecular model is presented whereby hypoxia, inflammation, and bone morphogenetic protein (BMP)–dependent signaling upregulate miR-21 in the pulmonary vasculature. In response, miR-21 represses Rho-kinase activation and, perhaps, other pathways to modulate the development of PH in vivo. BMPRII indicates bone morphogenetic protein receptor type 2.

regard for the interconnectivity of the gene sets appears suboptimal in identifying miRNA that target multiple related genes. In part, this may stem the wide variability in the manner in which data are curated from scientific reports, leading to literature-curated gene sets that are incomplete, biased, and perhaps dramatically different even if derived for the same disease. Thus, such gene sets may not always segregate into easily discernible functionally connected groups. For instance, we have curated a set of 46 genes from the scientific literature and expression arrays associated with the complex pregnancy-related disease preeclampsia. Unlike the PH network, these genes carried no discernible interconnectivity (39 genes were recognized in the consolidated interactome; largest connected component=1 without any edges), despite all genes being associated with preeclampsia. Thus, there exists little confidence in using this gene set for an analysis to capture miRNA that synergistically regulate discernible functional pathways (K.A. Cottrill and S.Y. Chan, MD, PhD, unpublished data, 2012). Such examples highlight the importance of first confirming a defined network of genes in order to then identify miRNA that regulate multiple targets in concert for a unified functional effect.

To date, the discovery of bona fide disease-modifying miRNA has depended on observations of altered levels of miRNA during disease manifestation. However, such an identification process can be limited by paucity of diseased human tissue samples, as in PH. In addition, unlike the *in silico* method presented here, it provides little insight into functional relationships among miRNA and cannot identify changes in miRNA function in the absence of direct alterations in miRNA expression. The predictive utility of this method should continue to improve with future variations in the design of this approach. Therefore, along with more traditional direct miRNA screening methods, we expect that network-based predictions will result in a more rapid and comprehensive identification of disease-modifying miRNA.

Previously, only 1 other miRNA, miR-204, has been rigorously studied in regard to its direct control of PH, effectively protecting against disease by targeting SHP2.[7] Significant changes in miR-21 were not appreciated in that study, potentially resulting from a relatively focused screen of a single cell type garnered from a narrow set of etiologic categories of PH. Conversely, our use of network biology predicts the importance of both miR-21 and miR-204 in PH as well as emphasizes their unique and shared targets in the PH network (Table II in the online-only Data Supplement). Thus, these findings emphasize the utility of such bioinformatics in expanding predictive power and suggest that miR-21 and miR-204 carry synergistic actions to coordinate protection against PH.

Finally, in contrast to a previous report describing downregulation of miR-21 in lung homogenate in PH,[44] our data unambiguously describe a pervasive, time-dependent upregulation of miR-21 in remodeled pulmonary vessels after injury in multiple rodent and human subjects suffering from end-stage PH (Figures 4 and 5). When one considers the well-established observations of enhanced hypoxic signaling and inflammatory IL-6 expression in rodent models of PH[45,46] and human disease,[47,48] such an upregulation of miR-21 in PH is mechanistically supported by the transcriptional induction of miR-21 by IL-6[49] as well as hypoxia (Figure 2 and Figure III in the online-only Data Supplement). Differences in RNA extraction from historical formalin-fixed lung samples or differences in the clinical context of disease may partially explain the contrasting observations of Caruso and colleagues.[44] Nonetheless, our findings, consistent in both reverse transcription polymerase chain reaction and in situ staining across several animal and human disease types, clearly demonstrate that miR-21 expression is increased most prominently during the latter stages of PH. Taken together with our findings of a protective role for miR-21 in PH, these observations also suggest that an inappropriate downregulation of miR-21 may predispose to PH. Consistent with this view, recent observations indicate that naturally occurring human mutations in the BMP pathway lead to decreased miR-21 expression.[25] These findings are consistent with reduced expression of miR-21 in *BMPR2*-haploinsufficient mice (Figure 2D), which are more susceptible to PH induced by hypoxia[24] and inflammation.[50] Thus, these data support a possible mechanism by which to explain how genetic deficiencies in BMP signaling predispose to pulmonary arterial hypertension: via decreased miR-21 expression in response to injury and loss of protection against disease progression.

In summary, a combination of *in silico* predictions, cell culture data, and animal modeling demonstrates that miR-21 integrates diverse pathogenic signaling to control the development of PH. Future attempts to combine network-based bioinformatics and biological validation may offer a more efficient and comprehensive approach to identifying addi-

tional miRNA important in PH and other complex diseases. Furthermore, the finding of such a central role for miR-21 provides much needed insight into the fundamental development of PH with broad implications for future therapeutic approaches.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Pulmonary hypertension (PH) is a deadly disease with diverse etiologies resulting in pathological dysregulation in the pulmonary vasculature. This dysregulation involves numerous intersecting signaling pathways, the coordinate control of which may depend on master molecular effectors. MicroRNA molecules (miRNA) are small, noncoding RNA that may act as such regulators, but their importance in PH is still unclear. In the present study, a unique network biology–based approach was used to identify microRNA-21 (miR-21) as 1 of a select group of miRNA predicted to control expression of a convergent set of pathways in PH. Underscoring the biologic plausibility of this finding, miR-21 is upregulated by hypoxia and bone morphogenetic protein receptor 2 signaling, 2 important PH triggers. In turn, miR-21 targets an important vascular effector, RhoB, and induces molecular changes in pulmonary endothelial cells consistent with vasodilation and decreased angiogenesis. Furthermore, miR-21 is upregulated in lung from humans with PH and from multiple rodent models of PH. In 1 of these models, the genetic absence of miR-21 causes accelerated disease progression. Taken together, these data identify miR-21 as a coordinate regulator of PH pathology that acts as a homeostatic brake to stave off PH progression. This study reinforces the critical role for miRNA in PH and introduces the utility of a network-based method for identifying additional groups of disease-modifying miRNA. Because complex interactions among these miRNA with their common targets will dictate their individual impact on disease, the study of such miRNA networks may offer insight into their utility as therapeutic targets.