The MicroRNA-130/301 Family Controls Vasoconstriction in Pulmonary Hypertension

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Background: The microRNA-130/301 family regulates pulmonary hypertension (PH), but its breadth of activity remains undefined.

Results: Predicted by network analysis, microRNA-130/301 members regulate vasoactive factors such as endothelin-1 for pulmonary vascular cross-talk.

Conclusion: The microRNA-130/301 family promotes vasoconstriction in PH.

Significance: This microRNA-based mechanism of vascular cross-talk is central to the systems-wide actions of microRNA-130/301 in PH.

Pulmonary hypertension (PH) is a complex disorder, spanning several known vascular cell types. Recently, we identified the microRNA-130/301 (miR-130/301) family as a regulator of multiple pro-proliferative pathways in PH, but the true breadth of influence of the miR-130/301 family across cell types in PH may be even more extensive. Here, we employed targeted network theory to identify additional pathogenic pathways regulated by miR-130/301, including those involving vasomotor tone. Guided by these predictions, we demonstrated, via gain- and loss-of-function experimentation in vitro and in vivo, that miR-130/301-specific control of the peroxisome proliferator-activated receptor γ regulates a panel of vasoactive factors communicating between diseased pulmonary vascular endothelial and smooth muscle cells. Of these, the vasoconstrictive factor endothelin-1 serves as an integral point of communication between the miR-130/301-peroxisome proliferator-activated receptor γ axis in endothelial cells and contractile function in smooth muscle cells. Thus, resulting from an in silico analysis of the architecture of the PH disease gene network coupled with molecular experimentation in vivo, these findings clarify the expanded role of the miR-130/301 family in the global regulation of PH. They further emphasize the importance of molecular cross-talk among the diverse cellular populations involved in PH.

Pulmonary hypertension (PH) is a complex vascular disease, characterized by hyperproliferation and vasoconstriction throughout the pulmonary vasculature. Severe forms of the disease, such as pulmonary arterial hypertension, display extensive vascular remodeling across multiple cell types, including pulmonary artery endothelial cells (PAECs), pulmonary artery smooth muscle cells (PASMCs), and fibroblasts (1). Pathogenic cross-talk between these cell types has been demonstrated in PH, as exemplified by the dysregulated release of paracrine growth factors (e.g. vascular endothelial growth factor (VEGF)) (2) or vasoactive mediators (e.g. endothelin-1 (EDN1) and serotonin) (3) from diseased PAECs (4). However, a comprehensive and coordinated program of regulation for these factors has yet to be fully characterized. Additionally, whereas cellular abnormalities, such as hyperplasia, occur across several vascular cell types, the degree of intercellular cross-talk required to orchestrate these phenotypic changes remains unknown (2).

Previously, we utilized a network-based approach to investigate the role of microRNAs (miRNAs) in the integrated control of PH pathogenesis. We identified the hypoxia-induced miR-130/301 family (miR-130a, miR-130b, miR-301a, and miR-301b) as a regulator of pulmonary vascular proliferation via control of a target gene, peroxisome proliferator-activated receptor γ (PPARγ) and downstream subordinate miRNA pathways (5). Each individual miR-130/301 family member is ubiquitously expressed and is typically up-regulated modestly in hypoxia. However, unlike other hypoxia-induced miRNAs in PH, such as miR-210, that are expressed independently of others (6, 7), the coordinated up-regulation of miR-130/301 as a family defines their robust biology because one family member alone does not account for the endogenous effects of this miRNA cohort (5). In vivo, pulmonary delivery of miR-130a (in combination with the VEGF receptor antagonist SU5416) promoted PH in mice, as reflected by pulmonary vascular remod-
MicroRNA-130/301 Family Promotes Pulmonary Vasoconstriction

Ebling and right ventricular systolic pressures. Conversely, pharmacologic inhibition of miR-130/301 in a mouse model of PH (chronic hypoxia with serial administration of SU5416 (8)) ameliorated such indices of PH. However, although we demonstrated the proliferative actions of miR-130/301, we only validated a small minority of the target genes and pathways predicted for this miRNA family. Thus, the true breadth of influence by the miR-130/301 family in PH may be even more extensive. There exists, for example, a great diversity of PPARγ function in vascular biology beyond proliferation, from vasoconstriction to metabolism to extracellular matrix deposition (9–12). In PH, a link has been reported between the vasoconstrictive factor EDN1 and PPARγ (13, 14), and in turn, EDN1 is known to promote vascular smooth muscle cell contraction and increase vasoconstriction in multiple forms of rodent and human PH (3, 15–17). However, multiple molecular pathways can regulate EDN1 expression (14, 18, 19) and other effectors of vasomotor tone, and the comprehensive mechanisms by which these actions are coordinated in PH are still undefined.

Here, to identify additional pathogenic pathways that may be regulated by these miRNAs, we performed targeted network-based analyses of PH-relevant targets of the miR-130/301 family and their closest interactors. Guided by these predictions, we demonstrated that the miR-130/301–PPARγ axis regulates a panel of vasoactive factors, most notably EDN1, serving as central mediators of vascular communication between diseased PAECs and PASMCs, vasomotor tone, and PH manifestation in vivo.

EXPERIMENTAL PROCEDURES

Network Analyses—A PH disease network was constructed based on a combination of literature-based curation and network expansion in silico, as described previously (5). Network edges were based on functional molecular associations curated from a variety of human gene and molecular interaction databases (20). MicroRNA target prediction was performed using the TargetScan 6.2 algorithm (21). The PH-relevant functions of the miR-130/301 family were assessed via a pathway enrichment analysis of a subnetwork of predicted miR-130/301 targets and their first degree interactors within the PH network. Pathway enrichment was performed using the Reactome Fl plug-in for the Cytoscape 3.1.1 environment, which compiles functional pathways from the Reactome, Biocarta, NCBI PID, and KEGG databases (22–24). Preferential expression by cell type of genes in the PH network was assessed using the human gene expression atlas data set (25), available through the BioGPS Web tool (26).

Cell Culture Reagents—Primary human PAECs and primary human PASMCs were purchased and propagated in EGM-2 cell culture media and SmGM-2 cell culture media (Lonza), respectively. Experiments were performed at passages 3–6. HEK293T cells (American Type Culture Collection) were cultured in DMEM containing 10% fetal bovine serum (FBS). HEK293T cells (American Type Culture Collection) were cultured in DMEM containing 10% fetal bovine serum (FBS). Human PASMCs were purchased and propagated in EGM-2 cell culture media and SmGM-2 cell culture media (Lonza), respectively. Experiments were performed at passages 3–6.

Lentivirus Production—HEK293T were plated and transfected 24 h later at 70–80% confluence using Lipofectamine 2000 (Invitrogen) with lentiviral plasmids and packaging plasmids (pPACK, System Biosciences), according to the manufacturer’s instructions. Virus was harvested, sterile filtered (0.45 μm), and utilized for subsequent gene transduction of PAECs or PASMCs (24–48 h incubation).

Oligonucleotides and Transfection—Pre-miRNA oligonucleotides (pre-miR-130a and negative control pre-miR-NC1 and premiR-NC2), anti-miRNA oligonucleotides (anti-miR-130a and anti-miR-NC), and custom designed tiny locked nucleic acid (LNA) oligonucleotides (tiny-130 (5′-ATTGCAGCT-3′) and tiny-NC (5′-TCATACTA-3′)) were purchased from Invitrogen/Ambion, ThermoScientific/Dharmacon, and Exiqon, respectively. siRNAs for PPARγ, c-JUN, and scrambled control were purchased from Santa Cruz Biotechnology, Inc. PAECs and PASMCs were transfected 24 h after plating at 70–80% confluence using Lipofectamine 2000 reagent (Invitrogen) and pre-miRNA (5 nM), anti-miRNA (20 nM), tiny LNA (20 nM), or siRNA (25 nM), according to the manufacturers’ instructions.

Plasmids—As described previously (5), coding sequence of PPARγ (BC006811) was amplified by polymerase chain reaction (PCR) using high fidelity polymerase Phusion (ThermoScientific) from an MGC cDNA clone (clone ID 3447380) and inserted in the pCDH-CMV-MCS-EF1-copGFP (System Biosciences) using Nhel and NotI restriction sites. The lentiviral parent vector expressing GFP was used as a control. Stable expression of these constructs in PAECs or PASMCs was achieved by lentiviral transduction. All cloned plasmids were confirmed by DNA sequencing.

Total RNA Extraction—Cells were homogenized in 2 ml of Qiazol reagent (Qiagen). Total RNAs, including small RNAs, were extracted using the miRNeasy kit (Qiagen) according to the manufacturer’s instructions. Total RNA concentration was determined using an ND-1000 microspectrophotometer (Nanodrop Technologies).

Quantitative RT-PCR of Messenger RNAs—CDNAs were generated by retrotranscription of messenger RNAs using a Multicript RT kit (Invitrogen). cDNAs were amplified via fluorescently labeled Taqman primer sets using an Applied Biosystems 7900HT fast real-time PCR device, as we described previously (27). -Fold change of RNA species was calculated using the formula (2^-ΔΔCt), normalized to actin expression.

Immunoblotting and Antibodies—As described previously (5), cells were lysed in radioligand precipitation assay buffer (Santa Cruz Biotechnology), and the protein concentration was determined using a Bradford assay (Bio-Rad). Protein lysate (40 μg) was resolved by SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad). Membranes were blocked in 5% nonfat milk in TN buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl) and incubated in the presence of the primary and then secondary antibodies. After washing in TN buffer containing 0.1% Tween, immunoreactive bands were visualized with the ECL system (Amersham Biosciences). Primary polyclonal or monoclonal antibodies to EDN1 (sc21625), NOS3 (sc654), PPARγ (sc7273), and VEGFA (sc152) were obtained from Santa Cruz Biotechnology. Primary antibodies for STAT3 (catalog no. 9139), P-STAT3 (Tyr-705; catalog no. 9145), and c-JUN (catalog no.
9165) were purchased from Cell Signaling. Primary antibody against actin (ab 3280) was obtained from Abcam. Appropriate secondary antibodies (anti-rabbit, anti-goat, and anti-mouse) coupled to HRP were used (Santa Cruz Biotechnology).

**Immunofluorescence**—Following treatment, cells were fixed with PBS/paraformaldehyde (4%) for 10 min and permeabilized with PBS/Triton X-100 (0.1%) for 10 min. Cells were then incubated with anti-PPARγ (sc7273; 1:50; Santa Cruz Biotechnology) at 4 °C overnight. Secondary antibodies coupled with Alexa-594 (Invitrogen) were used at 1:500. Nuclei were counterstained with DAPI (Sigma).

**Quantification of Endothelin-1 by ELISA**—Twenty-four hours after transfection, PAECs were washed in PBS, and growth medium was replaced by medium without serum. Twenty-four hours later, conditioned media from transfected PAECs were collected, and mature endothelin-1 was measured by ELISA (Enzo Life Sciences), according to the manufacturer's protocol. Mature endothelin-1 levels were also quantified in human and mouse plasma samples (see below) by ELISA.

**Contraction and Co-culture Assays**—PASMCs (50,000) were embedded in 100 μl of matrix gel as described previously (28, 29) and plated into a well of a 96-well plate. Briefly, cells were embedded in a mixture of growth factor reduced Matrigel (BD Biosciences) and collagen-I (BD Biosciences). Collagen-I solution was neutralized on ice with 0.1M NaOH in PBS and adjusted with 0.1N HCl to bring the pH of the solution to 7.5.

The collagen-I solution was then mixed on ice with Matrigel to obtain a final concentration of 1.5 mg/ml. Cells were trypsinized, counted, and resuspended in growth medium; then 1 volume of cells was mixed with 1 volume of the ECM mix. After 1 h at 37 °C, these matrices were overlaid with 100 μl of conditioned PAEC serum-free medium (transfected with siRNA, miRNA, or anti-miRNA and supplemented with ambrisentan (10 μM)). Every 12 h, medium was changed, and at day 4, the gels were photographed, followed by measurement of the relative diameter of the well and the gel using ImageJ software (National Institutes of Health). The percentage contraction was calculated using the formula, 100 × (well diameter − gel diameter)/well diameter. For co-culture experiments, conditioned media from PAECs transfected as indicated were applied on PASMCs or fibroblasts previously exposed to serum-free media for 24 h. Cells were collected at different times and analyzed by immunoblotting.

**Tissue Harvest**—After physiological measurements and prior to harvesting cardiopulmonary tissue, blood was collected by cardiac puncture, and plasma was prepared as described previously (5). Blood cells were then removed from the pulmonary vessels by right ventricular perfusion with 1 cm³ of saline. Organs were then harvested for histological preparation or flash-frozen in liquid N2 for subsequent homogenization and extraction of RNA and/or protein. To further process lung tissue specifically, prior to excision, lungs were flushed with PBS at constant low pressure (~10 mm Hg) via right ventricular cannulation, followed by tracheal inflation of the left lung with 10% neutral buffered formalin (Sigma-Aldrich) at a pressure of ~20 cm H₂O. After excision and 16 h of fixation in 10% neutral buffered formalin at 25 °C, lung tissues were paraffin-embedded via an ethanol-xylene dehydration series before being sliced into 5-μm sections (Hypercenter XP System and Embedding Center, Shandon).

**Immunohistochemistry of Mouse Lung**—Tissue samples (5-μm paraffin-embedded sections) were processed and prepared for standard histological and immunohistochemical procedures (5). The antibody used was goat anti-endothelin-1 (sc-21625, Santa Cruz Biotechnology). Pictures were obtained using an Olympus Bx51 microscope (×40 objective). Small pulmonary vessels (<100-μm diameter) present in a given tissue section (>10 vessels/section) that were not associated with bronchial airways were selected for analysis (n > 5 animals/group). Intensity of staining was quantified using ImageJ software (National Institutes of Health).

**Ethical Approval and Human Study Participants**—All experimental procedures involving the use of human plasma were approved by the Partners Healthcare Institutional Review Board. Ethical approval for this study conformed to the standards of the Declaration of Helsinki. Informed consent was obtained for right heart catheterization and blood sampling. For plasma harvest and analysis, human subjects were chosen with clinically significant dyspnea and undergoing right heart catheterization. Subjects were stratified by the presence or absence of clinical PH, as defined by elevated mean pulmonary arterial pressure >25 mm Hg (mean PAP).

**Human Plasma Sampling**—As described previously (5), patient blood was collected in standard Vacutainer tubes with K⁺-EDTA anticoagulant from the main pulmonary artery during right heart catheterization procedures. Plasma was extracted after standard centrifugation of blood, followed by storage at −80 °C.

**Forced Expression of miR-130a in Mouse Lung**—All animal studies were performed in accordance with institutional guidelines at the Brigham and Women’s Hospital and the University of Toronto. Eight-week-old mice (C57Bl6) were injected with SUG416 weekly (20 mg/kg; Sigma-Aldrich), accompanied by four serial intrapharyngeal injections (weekly dosing) of 100 μl of PBS solution containing 5% Lipoectamine 2000 (Invitrogen) mixed with 1 nmol of miR-control (pre-miR-NC) or miR-130a (pre-miR-130a). At day 24 (3 days after the last injection), right heart catheterization was performed as described previously (27), followed by harvest of lung tissue, as described above. Rosiglitazone was used at a concentration of 20 mg/kg/day. Briefly, rosiglitazone (Cayman Chemicals) was dissolved in 0.25% carboxymethyl cellulose medium viscosity aqueous solution. This rosiglitazone solution versus vehicle control was delivered daily by oral gavage to mice for 3 weeks.

**Inhibition of the miR-130/301 Family in Mouse Lung**—Eight-week-old mice (C57Bl6) were injected with SU5416 weekly (20 mg/kg; Sigma-Aldrich), followed by exposure to normobaric hypoxia (10% O₂; OxyCycler chamber, Biospherix Ltd., Redfield, NY) for 2 weeks. After 2 weeks and confirmation of PH development in five mice (right heart catheterization), mice were further treated with weekly SU5416 and three intrapharyngeal injections (every 4 days) of control or miR-130/301 shortmer oligonucleotides, designed as fully modified antisense oligonucleotides complementary to the seed sequence of the miR-130/301 miRNA family (10 mg/kg; Regulus). Specifically, the control and miR-130/301 shortmer oligonucleotides were
MicroRNA-130/301 Family Promotes Pulmonary Vasoconstriction

non-toxic, lipid-permeable, high affinity oligonucleotides. As described previously (5), the miR-130/301 shortmer carried a sequence complementary to the active site of the miR-130/301 miRNA family, containing a phosphorothioate backbone and modifications (bicyclic sugar) at the sugar 2' position. Three days after the last injection, right heart catheterization was performed, followed by harvest of lung tissue for RNA extraction or paraffin embedding, as described above.

Vascular Reactivity Assessment—Isolated perfused mouse lungs were prepared as described previously (4). In brief, male mice were anesthetized by an intraperitoneal injection of aver- tin (200 mg/kg) and placed in a water-jacketed chamber heated to 37 °C (isolated perfused lung size I; Hugo Sachs Elektronik, March-Hugstetten, Germany). Following tracheotomy and heparinization, the lungs were prepared for perfusion. After placement of a suture around the ascending aorta and the main pulmonary artery, a stainless steel cannula (1-mm inner diam- eter) was inserted into the pulmonary artery via the right ventricle and fixed by suture ligation. A second stainless steel can- nula was inserted into the left atrium via the apex of the left ventricle across the mitral valve to drain pulmonary venous efflux. Lungs were perfused at a flow rate of 50 ml/kg/min using a peristaltic pump (Ismatec Laboratoriumstechnik GmbH, Wertheim-Monfeld, Germany) with Hanks' balanced salt solu- tion containing 20% bovine serum albumin. The left atrial pres- sure was held at 2.7 cm H2O, and PAP was measured using saline-filled membrane pressure transducers (Hugo Sachs Elektronik, March-Hugstetten, Germany).

Statistics—Cell culture experiments were performed at least three times and at least in triplicate for each replicate. The number of animals in each group was calculated to measure at least a 20% difference between the means of experimental and control groups with a power of 80% and S.D. of 10%. The num- ber of unique patient samples for this study was determined primarily by clinical availability. RT-qPCR on human plasma, in situ expression/histologic analyses of mouse tissue, and pulmo- nary vascular hemodynamics in mice were performed in a blinded fashion. Numerical quantifications for in vitro experi- ments using three-dimensional cultured cells or in situ quanti- fications of transcript/miRNA expression represent mean ± S.D. Numerical quantifications for physiologic experiments using mice or human reagents represent means ± S.E. Immuno- blot images are representative of experiments that have been repeated at least three times. Micrographs are representative of experiments in each relevant cohort of mice. Paired samples were compared by Student’s t test. Comparison of multiple samples was performed by analysis of variance, followed by Student Newman-Keuls post hoc testing. Correlation analyses were performed by Pearson correlation coefficient calculation, as described previously (30).

RESULTS

Network Analysis in Silico Predicts That the miR-130/301 Family Targets a Range of PH-relevant Functional Pathways, Including Vasomotor Tone—In our prior study (5), we utilized in silico network principles to identify the pro-proliferative actions of the miR-130/301 family in PH. Beyond proliferation, additional functions of the miR-130/301 family are suggested by this family’s broad cohort of predicted targets (31, 32) as well as the known pleiotropy of the miR-130/301 target PPARy in vascular biology as a whole (9, 33). Here, we chose to cast a wider net of investigation, instead focusing on entire functional pathways predicted to be under the control of the miR-130/301 family. Selection of individual miRNA targets for study based on unfiltered pathway enrichment alone can be an inefficient and sometimes misleading method to determine actions most relevant to PH. This is especially evident in the case of miR-130/ 301, where the entire gamut of predicted targets (16 predicted targets in a curated list of known PH “seed” genes, as we described previously (27)) spans more than 50 distinct canonical pathways (Fig. 1A and Table 1). Many of these individual pathways may be cell type-specific or context-specific and thus not directly relevant to PH (e.g. pathway 13, axon guidance). Furthermore, a priori study of single target genes without regard for the entire network of miRNA targets increases the likelihood of focusing solely on regulatory mechanisms that modulate the “fine tuning” of gene expression while entirely ignoring those processes that orchestrate robust biology.

To improve our ability to identify functional pathways and targets under the greatest systems-level control by the miR- 130/301 family, our in silico model of the PH disease gene net- work was further analyzed. As described previously (5), this network was constructed using a combination of literature- based curation and network expansion in silico, resulting in a set of 249 genes with known and predicted relevance to PH pathogenesis. Network edges were based on functional molecular associations curated from a variety of human gene and molecular interaction databases (20). We first compared our PH network genes against the TargetScan 6.2 miRNA target prediction algorithm (21) in order to generate a comprehensive set of PH-relevant miR-130/301 family targets. These targets and their first degree interactors covered more than 70% of the PH network (177 genes), emphasizing the global role of the miR-130/301 family in the regulation of this disease. We also compared the genes in our subnetwork against a publically available atlas of human primary cell gene expression data sets (25), isolating those miR-130/301 targets (28 in total) and inter- actors that are expressed to a moderate or high degree in PH- relevant cell types (Fig. 1B). Notably, most of the PH-relevant target genes showed moderate to high expression in at least two, and mostly three, pulmonary vascular cell types relevant to PH (endothelial, smooth muscle, and fibroblast), correlating with the ubiquitous expression of this miRNA family and its broad actions throughout the vasculature. Notably, rare miR- 130/301 targets were preferentially expressed at high levels in each of these cell types, most notably including EDN1 in endo- theelial cells. Thus, this miRNA family may carry unique cell type specificity of function as well as a shared program of actions inherent across cell types.

To assess functional pathway enrichment in the context of the entire PH-relevant miR-130/301 targets, the pool of miR- 130/301 targets and interactors was catalogued into pathway subnetworks, based on several curated pathway databases, including the Kyoto Encyclopedia of Genes and Genomes (KEGG), Reactome, Biocarta, and the NCI Pathway Interaction Database (NCI PID) (22–24). Importantly, these subnetworks...
FIGURE 1. The miR-130/301 family is predicted to regulate several functional pathways in the PH network. A, 16 targets of the miR-130/301 family were present in a curated set of 129 PH-relevant genes. These targets were cross-referenced with the KEGG, Biocarta, Reactome, and NCI PID pathway databases (22–24, 50) and were predicted to participate in 55 distinct functional pathways. Number labels are sized relative to the number of participating genes in each pathway. (Pathways are indexed in Table 1). B, genes in the PH network were sorted according to their preferential expression in each of three PH-relevant vascular cell types. Genes grouped under a given cell type displayed moderate to high expression in that context. C, miR-130/301 family targets (circled) and their first-degree interactors within the PH network cover several broad functional modules. Genes were cross-referenced with 24 PH-relevant pathways defined by the union of multiple functionally related gene sets in the KEGG, Biocarta, Reactome, and NCI PID pathway databases (22–24, 50) (see also Table 2). EDN1 (enlarged) is a unique target in the “vasomotor tone” module, given its specificity to the vascular endothelium (as highlighted in B) and its known importance as a secreted vasoconstrictive factor in PH.
encompassed several broad modules of related functional pathways (with overlap), the largest of which are detailed in Fig. 1C (see also Table 2), including vascular inflammation, vasomotor tone, and control of the hypoxic response. Although each targeted module may represent a valid component of the miR-130/301-mediated response (such as those encompassing “hub” genes SP1 and MAPK1, among others), we focused here on the targets and interactors that participate in vasoconstriction and control of vasomotor tone. These are inherently important in the control of vascular cell cross-talk and, along with the TGF-β signaling pathway, accounted for the largest contingent of the miR-130/301 target subnetwork. We chose to focus specifically on EDN1, given its identity as a predicted miR-130/301 target, its unique specificity to the vascular endothelium (as highlighted in Fig. 1B), and its known role as a secreted vasoconstrictive factor. Together, these in silico data predict the control of EDN1 and its related factors by the miR-130/301 family for intercellular communication and control of pulmonary vasomotor tone.

The miR-130/301 Family Regulates the Production of Vasoactive Factors Such as EDN1 in PAECs—In order to validate experimentally the predicted importance of miR-130/301 for vasomotor tone, we performed gain-of-function and loss-of-function experiments interrogating the regulation of a panel of downstream vasoactive factors known to be modulated in PH and all included in the module of genes related to miR-130/301-specific control of vasomotor tone (Fig. 1). In normoxic (21% O₂) PAECs, forced expression of miR-130a up-regulated vascular endothelial growth factor-A (VEGFA), whereas it down-regulated endothelial nitric-oxide synthase (NOS3) (Fig. 2, A and C), both factors that are not directly targeted by miR-130/301 but were included as first degree interactors in the module.
Furthermore, particularly prominent at the protein level (Fig. 2), reversal of the miR-130-dependent effects on EDN1 expression (Fig. 2, C) was more pronounced when miR-130/301 family members were endogenously inhibited, and during normoxia as well as hypoxia, EDN1 was more consistently controlled by miR-130/301 inhibition and forced expression of its respective target genes, indicating the importance of coordinated action by this miRNA family.

The level of regulation of EDN1 by this miRNA family was greater extent than that of the inhibitor miR-130a alone, emphasizing the importance of coordinated action by this miRNA family.

The level of regulation of EDN1 by this miRNA family was particularly notable. Both at the transcript and protein levels and during normoxia as well as hypoxia, EDN1 was more consistently controlled by miR-130/301 inhibition and forced expression (Fig. 2, A–C). Furthermore, released EDN1 levels were elevated in conditioned media after forced expression of miR-130a, corresponding with the intracellular increases of EDN1. These levels were reciprocally decreased by tinymer inhibition of miR-130a in hypoxia (Fig. 2D), again to a greater extent than inhibition of miR-130a alone. Similarly, in plasma derived from the pulmonary arteries of PH patients.
demographics as described previously (5)), a positive linear correlation was observed between circulating levels of EDN1 and individual miR-130/301 family members (Fig. 3 A). Circulating EDN1 levels were also correlated with the hemodynamic severity of disease, as assessed by mean PAP (mPAP) (Fig. 3 B). Taken together, these data suggest that elevated EDN1 expression in PH is associated with the activities of the miR-130/301 family.

The miR-130/301 Family Depends Critically on PPARγ for Control of Vasomotor Tone—The above data indicated that, despite carrying a transcript sequence putatively recognized by miR-130/301, EDN1 expression is controlled more robustly by alternate miR-130/301-specific activities. Notably, PPARγ is a separate direct target of this miRNA family also found in the constructed “vasomotor tone” gene module (Fig. 1), but its down-regulation may induce, rather than repress, EDN1. Specifically, prior reports have indicated that knockdown of PPARγ induced both PAEC proliferation and EDN1 up-regulation, whereas forced PPARγ expression under hypoxia attenuated these effects, suggesting a link between these factors (13). Given the up-regulation of EDN1 by miR-130/301, we investigated whether this relationship was under the control of the miR-130/301-PPARγ axis. First, in PAECs, we employed a lentiviral transduction system to express a PPARγ transgene that did not code for the miR-130/301 family binding site and thus was unaffected by this miRNA family. During forced expression of miR-130a in PAECs, PPARγ transgene expression reversed miR-130a-mediated alterations in EDN1 and NOS3 (Fig. 4, A and E). Similarly, in cells treated with the PPARγ agonist rosiglitazone, which activates the remaining pool of PPARγ (Fig. 4 B), these miR-130a-dependent changes in EDN1 and NOS3 were reversed (Fig. 4, C and E). Finally, siRNA knockdown of PPARγ altered EDN1 and NOS3 expression in the absence of stimulation by the miR-130/301 family (Fig. 4, D and E). Moreover, levels of secreted EDN1 in conditioned media were consistent with intracellular EDN1 expression in all cases (Fig. 4 F). In contrast, in all cases of PPARγ manipulation, VEGFA expression remained unchanged, relative to its expres-

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**FIGURE 2.** The miR-130/301 family regulates the production of vasoactive factors in PAECs. In normoxia (21% O2) or hypoxia (0.2% O2, 24 h), either forced expression of miR-130a mimic versus control (NC) or inhibition of miR-130a (Anti-miR-130a) versus inhibition of the entire miR-130/131 family (tiny-LNA-130) versus control (NC) was performed in cultured PAECs (A–D). RT-qPCR (A and B) and immunoblotting (C) revealed that forced miR-130a expression increased VEGFA (A and C) and EDN1 (A and C) and decreased NOS3 (A and C). Conversely, inhibition of the entire miR-130/301 family (tiny-LNA-130) reversed these gene expression changes compared with control (tiny-LNA-NC), particularly evident at the transcript level in hypoxic PAECs (B) and at the protein level in normoxic PAECs (C). In most cases, inhibition of the miRNA family was more effective than inhibition of miR-130a alone (Anti-miR-130a versus control (Anti-miR-NC), thus demonstrating the importance of the coordinated actions of this miRNA family. D, as assessed by ELISA, transfection by miR-130a (left graph) increased mature endothelin-1 expression in conditioned media of PAECs during normoxia (21% O2) and to a greater extent during hypoxia (0.2% O2). Alternatively (right graph), in hypoxia (0.2% O2) but not normoxia (21% O2), inhibition of the entire miR-130/301 family (tiny-LNA-130) decreased endothelin-1 expression to a greater extent than inhibition of miR-130a alone (Anti-miR-130a). In A and B, mean expression in control groups (miR-NC, anti-miR-NC, and tiny-LNA-NC) was assigned a -fold change of 1, with which relevant samples were compared. In all histograms, data are expressed as mean ± S.D. (error bars) (*, p < 0.05; **, p < 0.01).
tion in cells treated with miR-130a alone. Together, these findings reveal that PPARγ serves as a critical link between the miR-130/301 family and EDN1 and NOS3, key effectors controlling pulmonary vasomotor tone. However, other effectors under the control of the miR-130/301 family, such as VEGFA, are regulated independently of PPARγ, highlighting the importance of additional miR-130/301-mediated pathways in other aspects of vascular function.

Additionally, in agreement with our network predictions, which highlight a connection between EDN1, PPARγ, and AP1 (c-Fos/c-Jun heterodimer; Fig. 1C (Vasomotor Tone module) and Table 2 (under “Endothelin signaling”)), it has been previously reported that PPARγ negatively regulates the transcription of EDN1 by inhibiting AP1 binding on the EDN1 promoter region in vascular endothelial cells (34). To address how the miR-130/301-PPARγ axis controls EDN1, we hypothesized

FIGURE 3. Endothelin-1 is increased and correlates with the expression of miR-130/301 family members in plasma of PH patients. A, plasma levels of mature endothelin-1 were positively correlated with plasma levels of miR-130/301 family members in patient samples. Here, five non-PH controls (mPAP < 25 mm Hg) were compared with two groups of PH patients stratified by hemodynamic severity: seven patients with mPAP between 25 and 45 mm Hg and seven patients with mPAP of >45 mm Hg. B, plasma levels of mature endothelin-1 progressively increased with hemodynamic severity of PH, and patient stratification was performed as above. Error bars, S.E.
FIGURE 4. The miR-130/301 family modulates the production of specific vasoactive factors through PPARγ in PAECs. A, RT-qPCR revealed that miR-130a-dependent expression changes in EDN1 and NOS3, but not VEGFA, in PAECs were reversed by lentiviral transduction of a PPARγ (pPPARγ) transgene as compared with control (pGFP). In this context, normoxic PAECs were transfected with miR-130a or miR-NC. B, pharmacologic activation of PPARγ activity was achieved by 24-h treatment with rosiglitazone (10 μM). As compared with vehicle (DMSO), rosiglitazone (Rosi) activated the nuclear translocation of PPARγ in both miR-NC- and miR-130a-expressing cells. C, pharmacologic activation of PPARγ activity was achieved by 24-h treatment with rosiglitazone (10 μM). As compared with vehicle (DMSO), rosiglitazone (Rosi) reversed the miR-130a-dependent alterations in transcript expression of NOS3 and EDN1 but not VEGFA. D, similarly, transcript levels of VEGFA, EDN1, and NOS3 were quantified in normoxic (21% O2) or hypoxic (0.2% O2) PAECs 48 h after transfection with siRNA against PPARγ (si-PPARγ) or siRNA control (si-NC). Under both normoxia and hypoxia, such PPARγ knockdown altered EDN1 and NOS3, but not VEGFA, expression compared with si-NC. E, under the same conditions as in A–D, immunoblotting revealed corresponding alterations in protein level expression of EDN1, NOS3, and VEGFA following forced expression of PPARγ, treatment with rosiglitazone, and transfection with si-PPARγ. F, as assessed by ELISA, secreted EDN1 levels in conditioned media from PAECs treated as in A–D were decreased following lentiviral transduction of PPARγ and in the presence of rosiglitazone and increased 48 h after transfection with siPPARγ. In A–C, mean expression in control groups (miR-NC and si-NC) was assigned a -fold change of 1, with which relevant samples were compared. In all histograms, data are expressed as mean ± S.D. (error bars) (*, p < 0.05; **, p < 0.01).
that antagonizing c-Jun in PAECs would blunt the effects of either miR-130a overexpression or PPARγ knockdown on EDN1 expression. During forced expression of miR-130a in PAECs, down-regulation of c-Jun indeed blunted miR-130a-mediated alterations in EDN1, but not VEGFA, at both mRNA (Fig. 5A) and protein levels (Fig. 5C). Similar results were observed in the presence of si-PPARγ (Fig. 5, B and D). As such, c-Jun controls the miR-130/301-dependent and PPARγ-dependent transcription of EDN1 specifically, thus correlating with prior reports of PPARγ inhibiting AP1 binding on the EDN1 promoter.

The miR-130/301-PPARγ-EDN1 Axis Induces Paracrine Activation of STAT3 and Actinomyosin-dependent Contraction of PASMCs—Considering this regulation of EDN1 in PAECs, we hypothesized that the miR-130/301-PPARγ axis controls paracrine activation of contractile vascular function. STAT3, a transcriptional activator that acts in response to cytokine and growth factor stimulation, has been previously linked to proliferation, vasoconstriction, and resistance to apoptosis in PASMCs (29, 35, 36). Given the miR-130/301-PPARγ-mediated up-regulation of STAT3 we previously reported in PASMCs (5), we next investigated whether the miR-130/301-PPARγ-EDN1 axis might regulate PASMC function in a STAT3-dependent fashion. In a previously validated in vitro assay of cellular contraction (Fig. 6A) (28, 29), direct EDN1 exposure activated STAT3 activity in PASMCs, as demonstrated by increased STAT3 tyrosine phosphorylation (Tyr-705) (Fig. 6B) and increased actinomyosin contraction (Fig. 6C). Similarly, conditioned media from either PAECs expressing miR-130a or PAECs exposed to siRNA specific for PPARγ increased PASMC-specific STAT3 tyrosine phosphorylation (Fig. 7A) and actinomyosin contraction (Fig. 7D). Conversely, PASMC-specific STAT3 phosphorylation (Fig. 7A) and contraction (Fig. 7D) were abrogated by conditioned media har-

![FIGURE 5.](http://example.com/figure5.png) The miR-130/301 family controls the transcription of endothelin-1 through a specific PPARγ-AP1 (c-Fos/c-Jun) axis. A, in PAECs, RT-qPCR revealed that miR-130a-dependent alterations in EDN1, but not VEGFA, were reversed by siRNA knockdown of c-Jun as compared with siRNA control (si-NC). B, RT-qPCR revealed that PPARγ-dependent alterations in EDN1 were reversed by si-c-Jun as compared with control (si-NC). C and D, under the same conditions as in A and B, immunoblotting and densitometry quantification revealed corresponding alterations in EDN1 at the protein level. In A–D, mean expression in control groups (miR-NC + si-NC and si-NC + si-NC) was assigned a -fold change of 1, with which relevant samples were compared. In all histograms, data are expressed as mean ± S.D. (*, p < 0.05; **, p < 0.01). A.U. refers to arbitrary units.
MicroRNA-130/301 Family Promotes Pulmonary Vasoconstriction

FIGURE 6. Endothelin-1 activates STAT3 phosphorylation and actinomyosin-dependent contraction of PASMCs. A, schematic diagram of coculture experiments; conditioned media from transfected PAECs (miRNA, anti-miRNA, tiny-LNA, or siRNA under normoxic or hypoxic conditions) were filtered and applied onto naïve PASMCs cultured in Matrigel. At specific times, PASMCs or PASMC-Matrigel cultures were arrested and analyzed for STAT3 activation (phosphorylation) or contraction, respectively. B, immunoblotting revealed that exposure of PASMCs to endothelin-1 led to a time-dependent increase of STAT3 phosphorylation (P-STAT3). C, as a reflection of PASMC contractile function, application of endothelin-1 into a PASMC culture in Matrigel for 4 days led to Matrigel contraction as compared with vehicle control.

The miR-130-301-PPARγ Axis Regulates EDN1 Expression and Pulmonary Vascular Hemodynamics in Vivo—To determine whether this regulatory axis is active in vivo, a series of gain-of-function and loss-of-function experiments were performed to manipulate this miRNA family and its downstream effectors in a mouse model of PH, namely via treatment with the VEGF receptor antagonist SU5416 and chronic hypoxia (8). First, in the presence of SU5416, chronic pulmonary expression of miR-130a in wild type mice was studied in place of chronic hypoxia. miRNA was delivered via four weekly intrapulmonary injections of liposomally encapsulated miR-130a oligonucleotide mimics, as adapted from prior protocols (36). As we described previously, this protocol resulted in the up-regulation of miR-130a, with subsequent PPARγ suppression, in whole lung tissue and in small pulmonary vessels, thus leading to increased vascular STAT3 phosphorylation, vascular remodeling, and right ventricular systolic pressure (5). Such delivery of miR-130a increased mean pulmonary arterial pressure and also blunted the degree of hypoxic pulmonary vasoconstriction, as measured by a lesser increase in pulmonary arterial pressure following a switch from normoxic to hypoxic ventilation (Fig. 8A; measured from intact pulmonary tissue isolated ex vivo as described previously (4)). Correspondingly, similar to prior studies demonstrating that chronic hypoxia decreases the acute vasoconstrictive response to hypoxia via alterations in the balance of vasoactive factors, such as EDN1 (37, 38), we found that miR-130a up-regulated EDN1 under these conditions in both small pulmonary vessels and plasma (Fig. 8, B–D), an effect that was reversed by PPARγ activation via simultaneous daily administration of oral rosiglitazone.

Conversely, inhibition of the miR-130/301 family in the pulmonary vasculature was achieved by pharmacologic means. As described previously (5), after 2 weeks of treatment with hypoxia + SU5416, right ventricular systolic pressure elevation was confirmed, followed by two additional weeks of hypoxia + SU5416 in the setting of three serial intrapulmonary injections of either a fully modified, short anti-miR complementary to the seed sequence (e.g. “shortmer”) or scrambled control. In that setting, we previously reported a derepression of PPARγ, a decrease in phosphorylated STAT3, and protection against the histologic and hemodynamic consequences of PH. Here, we found that such delivery of short-130 prevented the blunting of pulmonary arterial reactivity in response to hypoxia (Fig. 8E). Correspondingly, EDN1 expression was decreased in both vascular tissue and plasma (Fig. 8, F–H). Thus, considering both gain-of-function and loss-of-function experimentation, we conclude that chronic induction of endogenous miR-130/301 family members is necessary and sufficient to increase pathogenic expression of EDN1 in vivo, leading to alterations of pulmonary vasoreactivity, and such robust actions depend, at least in part, upon hierarchical control of PPARγ.

DISCUSSION

By combining analysis of gene network architecture with direct experimentation, we define the importance of the miR-130/301 family and its direct target PPARγ in specific control of pulmonary vascular cell cross-talk and vasomotor tone. Specifically, we found that, in addition to vascular proliferation, this family regulates a panel of vasomotor effectors throughout the vasculature, allowing it to modulate intercellular communication between the disparate cell types involved in PH. Moreover, we defined the intricate control of EDN1, which, when up-regulated by miR-130/301, induces STAT3 activation and vasoconstriction in PASMCs (Fig. 9). Taken together, these data characterize the miR-130/301 family as master regulator of a hierarchy of pathogenic pathways beyond cellular proliferation relevant to the pulmonary vasculature and PH.

Although the pool of known PH-relevant genes and triggers is growing rapidly, our understanding of any type of a global regulatory structure in the context of PH has remained limited. Guided by network theory, we now provide proof of extensive control of PH by miR-130/301 that extends beyond vascular
proliferation alone (5) and impacts vasoconstriction. The utility of such a network-based approach is further supported by our discovery that miR-130/301-specific control of EDN1 depends on a much more complex regulatory network than merely direct targeting of EDN1 alone. In fact, despite predictions of a putative target sequence in the EDN1 transcript, the seemingly paradoxical response of net up-regulation of EDN1 by miR-130/301 (Fig. 2) is not an uncommon scenario in miRNA biology. In specialized contexts, miRNAs may induce expression of their gene targets (39). Alternatively, as in this case, indirect regulatory pathways exist (e.g. PPARγ-AP1; Fig. 5) that ultimately are more powerful stimuli on EDN1 expression than direct miR-130/301 engagement of the EDN1 transcript itself. Such complex regulatory features may serve as feedback rheostats for particularly well connected miRNA target genes. Nonetheless, TargetScan 6.2 carries a verified low false positive rate in predicting the involvement of gene targets in the biology of specific miRNAs (21). However, this algorithm and others like it can have difficulty in predicting the direction by which each specific target is modulated, especially when the entire network of miRNA targets is engaged. As such, the use of miRNA target prediction algorithms in isolation can lead to

FIGURE 7. The miR-130/301-PPARγ-EDN1 axis in PAECs induces paracrine activation of STAT3 and actinomyosin-dependent contraction of PASMCs. A, PASMCs were treated with conditioned media from transfected PAECs (miRNA, anti-miRNA, tiny-LNA, or siRNA under normoxic or hypoxic conditions). STAT3 phosphorylation in PASMCs increased in the context of media derived from miR-130a-expressing and PPARγ-silenced PAECs (normoxic conditioning, left and middle panels) but decreased in the context of media from PAECs where miR-130/301 was inhibited (hypoxic conditioning, right panels). B, the miR-130a-dependent increase of STAT3 phosphorylation in PASMCs was reversed by lentiviral transduction of a PPARγ (pPPARγ) transgene as compared with control (pGFP) in PAECs (left panels) or by PPARγ activation via rosiglitazone in PAECs (right panels). C, PASMCs were treated as in A with (+) or without (−) the endothelin receptor antagonist ambrisentan (10 μM). Notably, ambrisentan prevented STAT3 activation in the setting of either miR-130a expression (left panels) or PPARγ-silencing in PAECs, thus demonstrating the downstream importance of EDN1 in such PASMC activation. D and E, Matrigel contraction was analyzed after treatment of PASMCs as in A–C, demonstrating that STAT3-dependent PASMC contraction is directly driven by the miR-130/301-PPARγ-EDN1 axis in PAECs. In all histograms, data are expressed as mean ± S.D. (error bars) (*, $p < 0.05$; **, $p < 0.01$; NS, not significant).
MicroRNA-130/301 Family Promotes Pulmonary Vasoconstriction

A. mPAP (cmH2O)

Baseline

miR-NC (n=5)

miR-130a (n=4)

Delta mPAP (cmH2O) (Normoxia -> Hypoxia)

Mouse lung

B. EDN1

miR-NC

miR-130a

Veh.

Rosiglitazone

C. [EDN1] (pg/mL)

NC 130a

NC 130a

Rosiglitazone

D. Edn1

Actin

Densitometry (AU)

miR-NC

miR-130a

E. delta PAP (cmH2O) (Normoxia -> Hypoxia)

Mouse lung

F. EDN1

Normoxia

Ctrl (2 weeks)

Short-NC (n=7)

Short-130 (n=7)

E. delta PAP (cmH2O) (Normoxia -> Hypoxia)

Mouse lung

G. [EDN1] (pg/mL)

Ctrl (2 weeks) PBS

Short-NC

Short-130

hypoxia + SU5416

H. Edn1

Actin

Densitometry (AU)

Short-NC

Short-130

hypoxia + SU5416
MicroRNA-130/301 Family Promotes Pulmonary Vasooconstriction

In regard to the specific biology of PH, although it is known that diseased PAECs secrete a variety of growth and vasoactive factors, the extent of their contribution to PASMC dysfunction is not well understood (2). In that vein, our data position the miR-130/301 family at a key checkpoint coordinating the intercellular cross-talk between PAECs and PASMCs via controlling released vasoactive factors. Additionally, although our research has primarily focused on the PPARγ-dependent actions of the miR-130/301 family, we have also demonstrated its PPARγ-independent regulation of other vasoactive factors, such as VEGFA. Thus, this miRNA family may have other direct targets, outside of PPARγ, with immediate relevance to this disease.

In addition to their previously defined pro-proliferative actions in PH (5), miR-130/301 family members were also found here to control pulmonary arterial reactivity in vivo in response to chronic hypoxia (Fig. 8). Hypoxic pulmonary vasoconstriction (HPV) is a pulmonary-specific, acute vasomotor response of pulmonary resistance arteries to alveolar hypoxia that optimizes pulmonary gas exchange. Previously, blunted HPV (38) and an overall alteration of pulmonary vasoactivity in the chronically hypoxic lung (40) were described as hallmarksof hypoxic PH, depending critically upon a complicated balance of effectors controlling vasoconstriction and vasodilation. Functional connections exist among pathways underlying HPV and the chronic effects of vasoconstriction in PH, but the exact overlapping molecular mechanisms are incompletely understood. Importantly, the actions of miR-130/301 phenocopied the known effects of chronic hypoxia, attenuating HPV (38). Conversely, miR-130/301 shortmer oligonucleotides restored intact HPV, thus replicating the effects of reversing PH on HPV in prior studies (41). Previous work has implicated a loss of Kᵢ1,5 and Kᵢ2.1 channels in this impairment of HPV in chronic hypoxia (38). Here, we found that such vasomotor alterations were also associated with a miR-130/301-dependent repression of PPARγ, a molecule previously associated with the regulation of pulmonary vasomotor tone via serotonin signaling (42). Through repressing PPARγ, the miR-130/301 family also increased EDN1 levels and reduced endothelial nitric-oxide.

FIGURE 9. Proposed model. Network-based bioinformatics coupled with experimental interrogation identified the miR-130/301-PPARγ axis at a key checkpoint coordinating EDN1-specific intercellular cross-talk between PAECs and PASMCs to regulate pulmonary vasomotor tone. Coupled with previously identified proliferative actions (4), this model elucidates the complete systems-wide control of PH by the miR-130/301 family even beyond proliferation and vasomotor tone. Such analyses may also connect this miRNA family to other disease conditions and could identify critical miRNA-based commonalities across the spectrum of cardiovascular diseases and human disease in general that otherwise have been missed.

erroneous assumptions regarding the complex biology of whole gene networks. In part, the use of network techniques such as ours in combination with miRNA target prediction algorithms can clarify the often overwhelming details of complex miRNA regulatory programs in order to prepare for experimental validation. Future applications of network theory may prove valuable in elucidating the complete systems-wide control of PH by the miR-130/301 family even beyond proliferation and vasomotor tone. Such analyses may also connect this miRNA family to other disease conditions and could identify critical miRNA-based commonalities across the spectrum of cardiovascular diseases and human disease in general that otherwise have been missed.
ide synthase (NOS3) expression. Although the exact contribution of nitric oxide in HPV is still debated (43, 44), the miR-130-301-dependent increases of EDN1 are certainly consistent with the known “preconstricted” vasomotor state in the chronically hypoxic lung (40), thus further blunting the HPV response. Such results suggest a central role for the miR-130/301-PPARγ-EDN1 regulatory axis in both HPV and PH during chronic hypoxia, thus emphasizing the importance of this miRNA family and its downstream targets in both acute hypoxic responses and chronic adaptations of the pulmonary vasculature. Given the ubiquitous expression of this miRNA family (45–47) yet the anatomic specificity of HPV, future work will be important to determine whether the vasomotor actions of miR-130/301 are indeed dependent on particular tissue or physiologic contexts, similar to the specialized pulmonary vascular functions of other miRNAs, such as miR-21 (27).

From a clinical perspective, the up-regulation of circulating miR-130/301 in PH has been confirmed by multiple sources (including our group (5) and Wei et al. (48)), thus establishing this miRNA family as a potential biomarker of PH. The strong correlation between miR-130/301 family expression and the released levels of EDN1 further supports the notion of circulating miR-130/301 levels as prognostic markers of PH severity. Moreover, given the miR-130/301 dependence of EDN1 up-regulation, it may be reasonable to expect that miR-130/301 plasma levels increase even before the initiation of vasoconstriction and vascular hyperplasia, thus allowing this miRNA family to be detected in advance of symptom onset. This type of temporal separation between circulating miRNA expression and the expression of more traditional biomarkers has been demonstrated in other contexts, such as the early release of inflammatory miRNA into circulation following strenuous exercise (49), and may be useful in detecting disease before it has become severe. Finally, these results emphasize the compelling candidacy of the miR-130/301 family as a robust therapeutic target in PH, particularly when used in combination with a PPARγ agonist, an endothelin receptor antagonist, or other vasodilatory medications.

In summary, we have defined the vasomotor functions of this miRNA family central to the control of pulmonary vascular paracrine signaling in PH. These results set the stage for future work to provide structure to the growing pool of miRNA, genes, and pathways that make up the PH diseasestate. They also more firmly identify the miR-130/301 family as key molecular effectors of PH that may be exploited for much needed diagnostic and therapeutic gains.

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