Estrogen Metabolite 16α-Hydroxyestrone Exacerbates Bone Morphogenetic Protein Receptor Type II–Associated Pulmonary Arterial Hypertension Through MicroRNA-29–Mediated Modulation of Cellular Metabolism

Xinping Chen, PhD; Megha Talati, PhD; Joshua P. Fessel, MD, PhD; Anna R. Hemnes, MD; Santhi Gladson, MS; Jaketa French, BS; Sheila Shay, BS; Aaron Trammell, MD; John A. Phillips, MD, PhD; Rizwan Hamid, MD, PhD; Joy D. Cogan, PhD; Elliott P. Dawson, MS; Kristie E. Womble, BS; Lora K. Hedges, BS; Elizabeth G. Martinez, DO; Lisa A. Wheeler, BS; James E. Loyd, MD; Susan J. Majka, PhD; James West, PhD; Eric D. Austin, MD, MSCI

Background—Pulmonary arterial hypertension (PAH) is a proliferative disease of the pulmonary vasculature that preferentially affects women. Estrogens such as the metabolite 16α-hydroxyestrone (16αOHE) may contribute to PAH pathogenesis, and alterations in cellular energy metabolism associate with PAH. We hypothesized that 16αOHE promotes heritable PAH (HPAH) via microRNA-29 (miR-29) family upregulation and that antagonism of miR-29 would attenuate pulmonary hypertension in transgenic mouse models of Bmpr2 mutation.

Methods and Results—MicroRNA array profiling of human lung tissue found elevation of microRNAs associated with energy metabolism, including the miR-29 family, among HPAH patients. miR-29 expression was 2-fold higher in Bmpr2 mutant mice lungs at baseline compared with controls and 4 to 8-fold higher in Bmpr2 mice exposed to 16αOHE 1.25 μg/h for 4 weeks. Blot analyses of Bmpr2 mouse lung protein showed significant reductions in peroxisome proliferator–activated receptor-γ and CD36 in those mice exposed to 16αOHE and protein derived from HPAH lungs compared with controls. Bmpr2 mice treated with anti–miR-29 (20-mg/kg injections for 6 weeks) had improvements in hemodynamic profile, histology, and markers of dysregulated energy metabolism compared with controls. Pulmonary artery smooth muscle cells derived from Bmpr2 murine lungs demonstrated mitochondrial abnormalities, which improved with anti–miR-29 transfection in vitro; endothelial-like cells derived from HPAH patient induced pluripotent stem cell lines were similar and improved with anti–miR-29 treatment.

Conclusions—16αOHE promotes the development of HPAH via upregulation of miR-29, which alters molecular and functional indexes of energy metabolism. Antagonism of miR-29 improves in vivo and in vitro features of HPAH and reveals a possible novel therapeutic target. (Circulation. 2016;133:82-97. DOI: 10.1161/CIRCULATIONAHA.115.016133.)

Key Words: estrogens ■ hypertension, pulmonary ■ metabolism ■ microRNAs ■ models, animal

Pulmonary arterial hypertension (PAH) is a progressive, devastating disease of the pulmonary vasculature that results in small-vessel occlusion and loss, increasing pulmonary vascular resistance and ultimately death resulting from right ventricular failure. The 2 strongest risk factors for disease are mutations in the gene bone morphogenetic protein receptor type II (BMPR2) and female sex. BMPR2 mutations are the major association with the heritable form of PAH (HPAH). Among most PAH subtypes, the female-to-male ratio is skewed to favor female patients ≈3:1. Despite major advances in understanding the development of PAH, curative therapies remain elusive, and it is unclear why female patients are diagnosed more frequently. The mechanisms of female predominance remain elusive, although an in-depth understanding may provide a major therapeutic opportunity.

Clinical Perspective on p 97

Received November 14, 2014; accepted October 2, 2015.
From Departments of Medicine (X.C., M.T., J.P.F., A.R.H., S.G., J.F., S.S., L.A.W., J.E.L., S.J.M., J.W.), Pharmacology (J.P.F.), Pediatrics (J.A.P., R.H., J.C., L.K.H.), and Pathology (E.G.M.), Vanderbilt University Medical Center, Nashville, TN; Department of Medicine, Baylor College of Medicine, Houston, TX (A.T.); and Bioventures, Inc, Murfreesboro, TN (E.P.D., K.E.W.).

The online-only Data Supplement is available with this article at http://circ.ahajournals.org/lookup/suppl/doi:10.1161/CIRCULATIONAHA.115.016133/-/DC1.

Correspondence to Eric D. Austin, MD, MSCI, Department of Pediatrics, Division of Pulmonary, Allergy, and Immunology Medicine, DD-2205 Medical Center N, Vanderbilt University School of Medicine, Nashville, TN 37232-2577. E-mail eric.austin@vanderbilt.edu

© 2015 American Heart Association, Inc.

Circulation is available at http://circ.ahajournals.org

DOI: 10.1161/CIRCULATIONAHA.115.016133
We and others have shown that an abnormal sex hormone milieu contributes to PAH risk and that modifying sex hormone exposures is a promising target. Estrogen production and metabolism is a complex process that changes over time and may occur in the sex-specific organs and peripherally for example, aromatase, the rate-limiting enzyme in the conversion of androgens to parent compound estrogens, is expressed in ovarian and extraovarian tissues, including the lung. It was recently shown that variations in genes that contribute to estrogen signaling associate with PAH. Intriguingly, pulmonary artery smooth muscle cells (PASMCs) from female patients with PAH express more aromatase compared with male patients, and aromatase inhibitor (anastrozole) treatment attenuates pulmonary hypertension (PH) in female rodent PH models. Although parent compound estrogens appear important to PAH, so too may be certain estrogen metabolites. BMPR2-associated HPAH in female patients associated with reduced expression of the estrogen metabolism gene CYP1B1, which may skew metabolism of parent estrogens into the estrogen metabolite 16α-hydroxyestrone (16αOHE). We demonstrated that preferential metabolism to 16αOHE associated with disease penetrance in HPAH. In addition, long-term 16αOHE exposure significantly increased PH penetrance and severity in murine models of Bmp2-associated PH. The deleterious effects of 16αOHE appear to involve both cellular processes and systemic abnormalities, including suppression of cellular BMP signaling and alterations in energy metabolism, as well as induction of pulmonary vascular injury and systemic insulin resistance. Thus, 16αOHE may promote cell-level alterations such as mitochondrial-metabolic abnormalities, which ultimately promote systemic metabolic abnormalities in PAH, including insulin resistance and hyperglycemia.

The pathogenesis of PAH is likely a complex process involving the interplay of multiple factors, including not only the circulating milieu but also environmental, genetic, and epigenetic factors that influence gene and protein expression. For example, there is emerging interest in the role of microRNA variation and activity in PAH. We sought to examine the relationship among 16αOHE-mediated estrogen signaling, altered energy metabolism, and PAH by first conducting unbiased microRNA expression arrays from the lung tissue of PAH patients and Bmp2 mutant mice. We discovered a shared upregulation in both species of the microRNA-29 (miR-29) family, which is known to regulate energy metabolism, and PAH by first conducting unbiased microRNA expression arrays from the lung tissue of PAH patients and Bmp2 mutant mice. We discovered a shared upregulation in both species of the microRNA-29 (miR-29) family, which is known to regulate energy metabolism, and that antagonism of miR-29 would attenuate PH in transgenic mouse models of Bmp2 mutation.

Methods

Human Study Population

Vanderbilt Pulmonary Hypertension Research Cohort study participants were recruited via the Vanderbilt Pulmonary Hypertension Center, whereas Vanderbilt Pulmonary Fibrosis Research Cohort study participants were recruited via the Vanderbilt Pulmonary Fibrosis Program. The Vanderbilt University Medical Center Institutional Review Board approved all study protocols. All participants or their surrogate custodians as appropriate gave informed written consent to participate in genetic and clinical studies. Heritable PAH (HPAH) lung tissue samples (n=2) were obtained after informed consent was obtained at the time of postmortem autopsy. PAH was defined either by autopsy results showing plexogenic pulmonary arteriopathy in the absence of alternative causes such as congenital heart disease or by clinical and cardiac catheterization criteria as previously published. HPAH was considered the type of PAH if a subject met 1 or both of the following criteria: family history of ≥2 subjects with confirmed PAH according to international standards of diagnostic criteria or detection of a mutation in a PAH-specific gene such as BMPR2. Idiopathic pulmonary fibrosis (IPF) lung tissue samples (n=2) were obtained after informed consent was obtained at the time of lung transplantation. IPF was defined according to the joint guidelines of the American Thoracic Society and European Respiratory Society. Both subjects had echocardiograms suggestive of normal right ventricular pressure, morphology, and function. Control lung tissue was isolated from donor lungs purchased commercially (n=2; Lonza, Allendale, NJ). The human lung donor subjects from all 3 groups were female.

RNA Extraction and Preparation

Total RNA was isolated from 10-μm tissue sections with the use of the miRNeasy mini-kit (Qiagen, Valencia, CA) according to the manufacturer’s guidelines. In brief, sample incubation was performed in Xylene at 50°C to remove excess paraffin. Samples were washed in ethanol. Proteins were degraded by digestion buffer with protease solution used as instructed. Isolation buffer and ethanol were used to bind samples to a spin-filter. DNase treatment was used to degrade DNA. The filter was washed several times, and total RNA was eluted in 60 μL elution solution. A spectrophotometer was used to check total RNA quantity and quality (Nanodrop ND-1000, Thermo Scientific, Wilmington, DE). Before labeling and hybridization, samples were subject to RNA quality control to assess the integrity of the RNA, its content of small RNA, and its concentration with a Bioanalyzer (Agilent 2100 Bioanalyzer) and NanoDrop instruments.

MicroRNA Microarray Assays

Exiqon Services was used for microRNA array profiling. All experiments were conducted at Exiqon Services in Denmark. The quality of the total RNA was verified by an Agilent 2100 Bioanalyzer profile. Total RNA (250 ng) from sample and reference was labeled with Hy3 and Hy5 fluorescent label, respectively, with the miRCURY LNA microRNA Hi-Power Labeling Kit, Hy3/Hy5 (Exiqon, Vedbaek, Denmark) following the procedure described by the manufacturer. The Hy3-labeled samples and a Hy5-labeled reference RNA sample were mixed pair-wise and hybridized to the miRCURY LNA microRNA array 6th generation (Exiqon), which contains capture probes targeting all microRNAs for humans, mice, or rats registered in the miRBASE 16.0. The hybridization was performed according to the miRCURY LNA microRNA array instruction manual with a Tecan HS4800 hybridization station (Tecan Austria). After hybridization, the microarray slides were scanned and stored in an ozone-free environment (ozone level <2.0 ppb) to prevent potential bleaching of the fluorescent dyes. The miRCURY LNA microRNA array slides were scanned with the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc), and the image analysis was carried out with the ImaGene 9.0 software (BioDiscovery, Inc). The quantified signals were background corrected (Normex with offset value 10) and normalized with the use of the quantile normalization method, which we have found produces the best between-slide normalization to minimize the intensity-dependent differences between samples.

For murine tissue studies, the Exiqon miRCURY LNA microRNA array assays were performed after 4 weeks of gene activation in Rosa26-control and Rosa26-Bmp2 mice. The mice had received 4 weeks of either vehicle (polyethylene glycol) or 1.25 μg/h 16αOHE. Each array consisted of a pool of 3 mice, and 2 arrays were used per condition.

Log-transformed microRNA expression data have previously been shown to be normally distributed and thus were treated as such for current statistical analyses. MicroRNA probes with expression levels at the level of background were eliminated from array results.
provided by Exiqon. With these results (196 probe sets for human data, 303 probe sets for mouse data), principal components analysis and the creation of heat maps were done in JMP11 (SAS Institute).

Real-Time Reverse Transcriptase–Quantitative Polymerase Chain Reaction
Real-time reverse transcriptase (RT)–quantitative polymerase chain reaction (qPCR) was conducted on all samples to validate the microRNA profiling results. Specific miScript primer assays for miR-29a, miR-29b, and miR-29c (Qiagen, Valencia, CA) were used to verify the expression by qPCR. RT reactions were performed with miScript II RT kit (Qiagen). The reactions were in 20 μL total volume containing 100 ng total RNAs (including small RNAs), 1x miScript HiSpec buffer (1x miScript HiFlex buffer for RNU6B), 1x miScript Nucleics Mix, and 1x miScript Reverse Transcriptase Mix. The reactions were incubated for 1 hour at 37°C and then for 5 minutes at 95°C. All RT reactions were run in duplicate. Real-time PCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems). The PCR included the RT product, miScript SYBR Green, and miScript primer assay (Qiagen). The reactions were incubated at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Relative expression was determined with the ΔΔCt method. RNU6B was used as the internal control according to manufacturer instructions and consistent with prior studies.47

Western Blot Analyses
Mouse or human lung tissue was homogenized in radioimmunoprecipitation assay buffer (PBS, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS) with protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO). Protein concentration was determined by BCA protein assay (Thermo Scientific, Rockford, IL). Primary antibodies used for Western blot included Glut4 (Abcam antibody), CD36 (Novus NB400-144), elastin (Abcam ab9514), peroxisome proliferator–activated receptor-γ (PPARγ; Abcam ab27649), and β-actin (Abcam ab8227).

Verification of miR-29 Direct Targets
The prediction of microRNA targets with bioinformatics algorithms has high false-positive and false-negative prediction rates.38 Although false-positive prediction can be excluded by experiments, false-negative predictions would miss true targets. To further explore miR-29 potential direct targets, we used a direct affinity-purification method using a microRNA pull-down assay followed by specific quantitative PCR using established protocols.39–41 In this approach, synthetic microRNA duplexes carrying a biotin group attached to the 3' end of the microRNA sense strand were transfected into murine pulmonary vascular smooth muscle cells. This resulted in the subsequent incorporation of the sense strand into the microRNA-induced silencing complex. After cell lysis, the microRNA-mRNA complexes were captured on streptavidin beads from which the mRNA was purified and analyzed with quantitative RT-PCR analysis of the affinity-purified mRNA. The PPARγ and HPRT were used as negative controls; in contrast, G6PC and HPRT were used as positive controls; in contrast, G6PC and HPRT were used as negative controls.

Animal Studies
Transgenic Mice
We used the previously described44–46 Rosa26-rTa2X TetO-\(\text{Bmp}^\text{2\text{Kd}}\)\(\text{FVB/N}\) and Rosa26-rTa2X TetO-\(\text{Bmp}^\text{2\text{Kd}}\)\(\text{delx4}\)\(\text{FVB/N}\) mice, called Rosa26-Bmp2\text{KdFVB/N}\) and Rosa26-Bmp2\text{KdFVB/N} for brevity. The term Rosa26 is used to identify control mice without Bmp2 mutation. R899X is an arginine-to-termination mutation at amino acid 899 in the BMPR2 tail domain found in the US33 family and several others,47 whereas Rosa26-Bmp2\text{KdFVB/N} has deletion from just after the transmembrane domain onward. For both Rosa26-Bmp2\text{KdFVB/N} and Rosa26-Bmp2\text{KdFVB/N}, expression of transgene occurs in all tissue types but only after the initiation of doxycycline. Both male and female mice were used.

For experiments on the effects of 16OHE exposure, adult Rosa26, Rosa26-Bmp2\text{KdFVB/N}, or Rosa26-Bmp2\text{KdFVB/N} mice had the transgene activated with doxycycline at 0.2 mg/g in chow. Mice were implanted with Alzet osmotic pumps delivering either vehicle alone (polyethylene glycol) or 16OHE at 1.25 μg/h, a dose we previously demonstrated to promote a PAH phenotype in these mice. This dose was initially demonstrated to be a dose at which 16OHE has significant estrogenic bioactivity in murine models.74 After 4 weeks with osmotic pumps, the mice underwent hemodynamic profiling and were euthanized and processed for microRNA purification.

We also performed experiments to antagonize miR-29 using an antagonim for miR-29 (α-miR29). LNA-anti-microRNAs were synthesized by Exiqon as an LNA-modified oligonucleotide that contained phosphorothioate backbone for use in microRNA functional studies. LNA–miR-29 was synthesized on the basis of the 5'-ATTTCAGAAGGTGCTGTA-3' sequence, which is complementary to miR-29. A scrambled oligonucleotide, LNA-Co, 5'-ACGCTCTACGCCCA-3', was chosen according to manufacturer instructions and used as control. The scrambled oligonucleotide was chosen because it met 3 requirements: (1) same nucleotide composition as the input sequence, (2) passed the same extensive sRNA filtering (eg, no low complex sequence), and (3) has the weakest (or no) match with any microRNA in the microRNA pool. LNA-anti-microRNAs were dissolved in saline and injected intravenously into mice via the tail vein once a week at a dose of 20 mg/kg. After 6 weeks, mice underwent hemodynamic phenotyping, as described below.

All animal procedures were approved by the Institutional Animal Care and Use Committee, Vanderbilt University School of Medicine (Nashville, TN).

Hemodynamic Phenotyping
Two-dimensional echocardiography was performed with a Vio770 High-Resolution Image System (VisuaSonics Toronto, ON, Canada). Echocardiograms, including B-mode, M-mode, and spectral Doppler images, were obtained the day before euthanasia with isoflurane anesthesia, as previously described.21

Right ventricular systolic pressure (RVSP) was directly measured via insertion of a 1.4F Mikro-tip catheter transducer (Millar Instruments, Houston, TX) into a surgically exposed right internal jugular vein as previously described.47 Pulmonary vascular resistance was calculated as (80×RVSP)/(3×cardiac output) as previously described.47

Immunostaining
Paraffin-embedded mouse lung sections were treated with a standard processing method for immunostaining and incubated with primary α-smooth muscle actin antibody (1:500; DAKO, Ft. Collins, CO) overnight, followed by Alexa 488 fluorescent secondary antibody (1:500; Invitrogen, Carlsbad, CA). All antibodies were diluted in a blocking buffer (Tris-buffered saline Tween with 10% FCS), and controls consisted of primary isotype with secondary antibody or secondary antibody only. Quantification of muscularization was performed by counting α-smooth muscle actin–positive vessels per field of view in paraffin-stained lung sections.

Immunolocalization of cermadines was performed on paraffin-embedded mouse lung tissue. Lung sections were deparaffinized and rehydrated. The sections were blocked with 5% normal goat serum or 5% BSA, followed by an overnight incubation at 4°C with cadherin antibody (Enzo Life Sciences, Farmingdale, NY). The next day, the sections were incubated with biotinylated IgM secondary antibody followed by incubation with horseradish peroxidase–conjugated streptavidin. Diaminobenzidine was used as a substrate for horseradish peroxidase (Vector Labs, Burlingame, CA). The sections were dehydrated and mounted in Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI) for light microscopic examination.
Chen et al 16αOHE Modifies HPAH via miR-29

Trichrome stain was performed on paraffin-embedded mouse lung sections. The trichrome stain reagents were purchased from Sigma-Aldrich, and the stain was performed according to manufacturer instructions.

Electron Microscopy

For electron microscopy studies, we used murine PASMCs and human endothelial-like cells derived from induced pluripotent stem cell lines as we previously described. Cells were cultured in 100-mm dishes and were subjected for LNA–miR-Co or LNA–miR-29 transfections. Twenty-four hours after transfection, the cells were fixed in 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer at room temperature for 1 hour and then transferred to 4°C overnight.

Specimens were processed for transmission electron microscopy and imaged in the Vanderbilt Cell Imaging Shared Resource-Research Electron Microscope facility. Briefly, the samples were postfixed in 1% osmium tetroxide at room temperature and then washed 3 times with 0.1 mol/L cacodylate buffer. Subsequently, the samples were dehydrated through a graded ethanol series, followed by incubation in 100% ethanol and propylene oxide and 2 exchanges of pure propylene oxide. Samples were embedded in epoxy resin and polymerized at 60°C for 48 hours. Ultrathin sections (70–80 nm) were then cut from the block and collected on 300-mesh copper grids. The copper grids were postsection stained at room temperature with 2% uranyl acetate and then with lead citrate. Samples were subsequently imaged on the Philips/FEI Tecnai T12 electron microscope at various magnifications.

Cells were randomly selected and pictured by a blinded observer in the electron microscopy core laboratory. All the mitochondria in the pictures were measured with tools from Image J, the open-source imaging program.

Insulin Resistance Measures

Insulin resistance was determined by the homeostasis model assessment of insulin resistance (HOMA-IR). This study was developed to model the dynamic interaction of insulin and glucose across a range of variations in insulin resistance and pancreatic β-cell function and correlates well with the degree of insulin sensitivity and resistance. The HOMA-IR uses the following formula: fasting glucose (mmol/L)×fasting insulin (μU/mL)/22.5. Mouse plasma was used for studies involving the HOMA-IR assay. Studies were performed by the Hormone Assay and Analytic Services Core at Vanderbilt University.

Statistics

Statistical methods for array analysis are described above. For non-array analyses with normal distribution, statistical significance was evaluated with the use of parametric statistical techniques with 2-sided testing, with a value of P<0.05 considered statistically significant. Statistical analysis was performed with the SPSS statistical package for Windows (version 21, IBM SPSS Statistics 21.0, Armonk, NY) or the JMP program (SAS Institute Inc, Cary, NC). Comparisons including multiple independent variables such as mutation status and 16αOHE or mutation status and presence of α-miR-29 were performed with 2-way ANOVA, with the Fisher least significant difference as a post hoc test to determine the source of significance.

For principal components analysis, data were first normalized so that the average for each probe set was zero, and then the principal components analysis function in JMP was applied to all microRNA assessed by the Exiqon arrays. Eigenvectors for the first and second principal components were exported and plotted for Figures 1A and 2A. Given the nature of the study, there was no adjustment for multiple comparisons.

Results

Arrays Suggest Disease-Specific Changes in MicroRNA Expression in Human PAH

MicroRNA expression arrays were used to search for differences in expression that might contribute to PAH pathogenesis, recognizing that end-stage lung disease may influence microRNA expression expression independently of PAH. Thus, Exiqon microRNA arrays were used to determine expression levels in female lungs from HPAH and control and from female IPF subjects (Table). Although IPF is also a global lung disease, the pathogenesis of IPF is distinct from that of PAH with the strict phenotypic selection we used. As a result, IPF subjects were included a priori as a mechanism to suggest whether any microRNA differences observed between HPAH and control lungs were a more generic result of global lung abnormality or a distinct feature of HPAH lungs not present in a separate lung disease (IPF).

The control subjects were healthy individuals who died in traumatic accidents. For each subject, 2 independent samples were taken, and for each disease state, a pooled set was run for comparison. Of 1439 microRNA probe sets, 196 were expressed above the noise in at least 1 IPF, PAH, or control subject. Principal components analysis showed that the 2 samples from each patient were nearly identical and that the 2 patients from each disease state clustered together closely (Figure 1A). Although the sample number is small, the low variability suggests a disease-specific effect. Comparing PAH and control shows that there were 65 probe sets with a 95% probability of at least a 30% change (Figure 1B). These included many probe sets described as regulating metabolism (marked by an asterisk), including the entirety of the miR29 cluster (miR-29a, miR-29b, and miR-29c). Upregulation of the miR-29 cluster was confirmed by quantitative RT-PCR with the use of RNA from control or PAH human lung (Figure 1C).

MiR-29 Is Induced by Both Bmpr2 Mutation and 16αOHE in Mice

We had previously published data suggesting that the primary pathogenic effect of estrogen in the context of BMPR2 mutation was related to deleterious effects on metabolism, including insulin resistance. We thus wished to determine whether the estrogen metabolite 16αOHE contributed to the regulation of microRNAs known to relate to metabolism, with particular interest in the miR-29 family.

To determine whether we could use our Bmpr2 mutant murine model of PH to model the microRNA dysregulation found in human patients, Exiqon microRNA arrays were used to determine microRNA expression levels in male Rosa26-Bmpr2ΔΔΔΔ transgenic mice. Male mice were used because they allow a more direct control of estrogen exposure so that the predominant estrogen exposure in the model system is 16αOHE. A pool of microRNA from 3 mice was used for each microRNA expression array to compare mice that received vehicle (n=3) with those receiving the estrogen metabolite 16αOHE (n=3) in osmotic pumps as previously described.

Of 1209 probe sets, 303 had expression levels above background level. Principal components analysis on them clearly separates the groups (Figure 2A), with the first principal component corresponding roughly to Bmpr2 mutation effect and the second principal component corresponding to 16αOHE effect, although the Bmpr2 mutant mice were already substantially moved on the 16αOHE effect axis. A heat map of the
The top 50 microRNAs with altered regulation showed the same microRNA cluster 29 in a group of genes with strong upregulation by 16αOHE in control mice and weaker upregulation in Bmpr2 mutant (microRNA labels with asterisks in Figure 2B). This was confirmed by quantitative RT-PCR (Figure 2C), which found that the miR-29 cluster (miR-29a, miR-29b, and miR-29c) had a roughly 2-time-higher expression in activated Bmpr2 mutant mice lungs at baseline, increasing to 4- to 8-times upregulation with 16αOHE treatment.

**Molecular Markers of Insulin Resistance Are Abnormal in Murine and Human PAH and Influenced by the Estrogen Metabolite 16αOHE and MiR-29**

As mentioned, we recently reported a potential causal relationship between increased 16αOHE and increased PAH penetrance, including alterations in insulin resistance–related pathways. Because we also recently found an association between glucocorticoid insensitivity and murine Bmpr2
PAH, we explored molecular markers of insulin resistance using the same mice (vehicle [n=6] versus estrogen metabolite 16αOHE [n=6]) as in the microarray experiment described above and shown in Figure 2 (male Rosa26-Bmpr2delx4+ transgenic mice; Figure 3).

Western blot analyses of whole mouse lung protein demonstrate that PPARγ and CD36 levels were each significantly reduced in wild-type animals exposed to 16αOHE compared with vehicle-treated wild-type animals. Bmpr2 mutant animals not exposed to 16αOHE had significantly reduced PPARγ and CD36 levels compared with vehicle-treated wild-type animals. Exposure of Bmpr2 mutant animals to 16αOHE further reduced both PPARγ and CD36 compared with Bmpr2 mutant animals treated with vehicle. These findings demonstrate that 16αOHE directly reduces PPARγ and CD36 levels and that the magnitude of reduction is higher in the setting of a Bmpr2 mutation (Figure 3A).

In addition, we analyzed the lung specimens from the human PAH subjects (n=2) evaluated by the whole lung microarray study described in Figure 1. Western blots show that PPARγ, GLUT4, and CD36 protein levels were substantially reduced in PAH patients (n=2, same as Figure 1) compared with control subjects (n=2, same as Figure 1; Figure 3B).

Next, pulmonary microvascular endothelial cells cultured from the male Rosa26-Bmpr2delx4+ transgenic mice were used to analyze in vitro protein production by endothelial cells. Cultured murine pulmonary microvascular endothelial cells from mice with the activated Bmpr2 mutation showed reduced levels of PPARγ, Glut4, and CD36 protein. These levels were further reduced by the addition of 16αOHE, consistent with the whole lung protein data (Figure 3C).

Because we suspect that metabolic irregularities are not cell specific, we believed that the mechanisms of interest would also be detectable in pulmonary vascular smooth muscle cells. We next hypothesized that antagonism of miR-29 would restore PPARγ and CD36 gene expression in vitro. Cultured mouse pulmonary vascular smooth muscle cells from male Rosa26-Bmpr2delx4+ transgenic mice were used to assess the effect of α-miR29. We first assessed the specificity of miR-29 antagonism. Metrics used were known gene targets that the miR-29 family downregulates. Addition of α-miR29 improved the expression of known targets, that is, CD36, Coll1a1, Eln, and peroxisome proliferator-activated receptor gamma, coactivator 1α (Ppargc1a), but not related genes, suggesting specificity of miR-29 antagonism (Figure 3D). Although alteration of gene expression is important, microRNA modulation of gene and protein expression is often not equal, particularly given that many microRNAs influence the expression of genes into proteins at the posttranscriptional level. The PPARγ and CD36 protein elevations on exposure to α-miR29 were confirmed by Western blot. Although CD36 protein was increased, PPARγ protein was significantly increased 14-fold in murine smooth muscle cell culture compared with control (Figure 3E).

**Table. Clinical Characteristics Relevant to PAH Diagnosis for Human Subjects**

<table>
<thead>
<tr>
<th>PAH-Related Phenotypic Data</th>
<th>HPAH Patient 1</th>
<th>HPAH Patient 2</th>
<th>IPF Patient 1</th>
<th>IPF Patient 2</th>
<th>CTL Patient 1</th>
<th>CTL Patient 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAH diagnosis age, y</td>
<td>20</td>
<td>13</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Current age, y</td>
<td>24</td>
<td>13</td>
<td>28</td>
<td>47</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Sex</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>NYHA functional class at diagnosis</td>
<td>3</td>
<td>3</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Baseline hemodynamic data at diagnosis</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Right atrial pressure, mm Hg</td>
<td>7</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Mean pulmonary artery pressure, mm Hg</td>
<td>66</td>
<td>2.5</td>
<td>12.5</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Responsive to acute vasodilator testing</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>PAH-specific therapies</td>
<td></td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Prostanoids</td>
<td>Yes</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphodiesterase-5 inhibitors</td>
<td>No</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelin receptor antagonists</td>
<td>No</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PAH patient 2 died of cardiopulmonary arrest before cardiac catheterization. PAH diagnosis was made by clinical and echocardiographic data and autopsy evaluation. CTL indicates control, HPAH, heritable pulmonary arterial hypertension; N/A, not available; NYHA, New York Heart Association; IPF, idiopathic pulmonary fibrosis; PAH, pulmonary arterial hypertension; and PVR, pulmonary vascular resistance.
UTR of CD36 (Figure I in the online-only Data Supplement) because miR-29a and miR-29c affinity purification did not pull down CD36 mRNA. These results suggest that PPARγ and CAV1, but not CD36, are direct targets of miR-29a and miR-29c.

Antagonism of miR-29 Significantly Improves Hemodynamics and Other Features of PH in a Murine Model of PH

Transgene-activated Bmpr2<sup>2998X</sup> mutant mice develop an anatomic, hemodynamic, and histological profile consistent with
PH, and the penetrance is increased by 16αOHE.\textsuperscript{22,44,45} We tested the hypothesis that miR-29 antagonism would prevent a pulmonary hypertensive phenotype in a study of animals of both sexes fed a Western diet. Bmpr2R899X mutant mice of both sexes (n=25: female, n=12; male, n=13) had significantly elevated RVSP with the transgene activated compared with controls (n=13: female, n=6; male, n=7). Weekly injections of α-miR29 for 6 weeks significantly reduced RVSP (Figure 5A) among the Bmpr2R899X mutation group (n=26: female, n=13; male, n=13) compared with controls (n=14: female, n=7; male, n=7), regardless of exposure to 16αOHE (squares) or vehicle (circles).

Other features of the hemodynamic profile were also improved by α-miR29 therapy. Pulmonary vascular resistance compared with controls (n=24: female, n=13; male, n=11) was elevated among Bmpr2R899X mutant mice without (n=11: female, n=5; male, n=5) and with (n=11: female, n=7; male, n=4) 16αOHE, as previously demonstrated (no α-miR29 exposure). Twenty-three Bmpr2R899X mice were exposed to α-miR29: 11 without 16αOHE (6 female, 5 male) and 12 with 16αOHE (6 female, 6 male). Pulmonary vascular resistance was significantly reduced among the Bmpr2R899X mutant mice (n=23) treated with α-miR29 compared with those not treated with α-miR29 (n=22); this was true for Bmpr2R899X mutant mice regardless of 16αOHE exposure (Figure 5B).

To assess histological changes associated with PH, the extent of pulmonary arterial muscularization was assessed. Control mice had significantly fewer muscularized small (<25-μm diameter) pulmonary arteries compared with Bmpr2R899X mutant mice. α-miR29 treatment reversed increased muscularization seen in Bmpr2R899X mice but did not change muscularization in control mice (Figure 5C and 5D; each symbol is an animal and each column represents n=4 per group, with 2 females and 2 males per each group of 4). Because miR29 downregulation has been associated with renal fibrosis, careful examination of the kidneys from mice exposed to α-miR29 treatment was conducted by a renal pathologist.\textsuperscript{54,55} There was no evidence of fibrosis on careful review (Figure II in the online-only Data Supplement).

### Antagonism of miR-29 Reverses the Lung Molecular Phenotype Suggestive of Insulin Resistance

Whole lung from Bmpr2R899X mutant mice have reduced PPARγ and CD36 protein levels compared with controls. Weekly α-miR29 treatment resulted in a significant increase in PPARγ and CD36 protein in both the control and Bmpr2R899X mutant mice (Figure 6A). In addition, HOMA-IR is elevated among Bmpr2R899X mutant mice. Control and Bmpr2R899X mutant mice treated with α-miR29 have a significant reduction in HOMA-IR; among Bmpr2R899X mutant mice, the level returns to that of controls (Figure 6B). We recently reported evidence for increased synthesis and deposition of ceramide associated with the PH phenotype in Bmpr2R899X mutant mice and sought to assess ceramide in the lungs with and without α-miR29 treatment.\textsuperscript{19} Ceramide is a key mediator of lipotoxicity elevated in the setting of insulin resistance.\textsuperscript{56} As expected, ceramide appeared elevated in the pulmonary vasculature of Bmpr2R899X mutant mice compared with controls, and α-miR29 treatment substantially reduced ceramide accumulation, consistent with an improvement in the molecular phenotype of insulin resistance (Figure 6C).

### α-MiR29 Treatment Increases Average Mitochondrial Size

Our group and others have been interested in the contribution of mitochondrial abnormalities to PH, and we previously...
showed that the global expression of a Bmpr2 mutation is sufficient to cause metabolic stress and mitochondrial oxidant injury, including excess production of mitochondrial-derived reactive oxygen species. This, in concert with the emerging appreciation that mitochondrial morphology is a dynamic process but that larger mitochondria may indicate reduced fission and associate with better outcomes in PAH, we sought to assess mitochondrial morphology with and without miR-29 antagonism.

One mechanism by which miR-29 antagonism may correct energy metabolic problems is through the regulation of mitochondrial fission and fusion; the contributions of mitochondrial dynamics to pulmonary vascular disease and right ventricular response to stress are of great interest in the field. The regulation of mitochondrial fission and fusion may occur via Ppargc1a or PPARγ increase, as suggested by Figure 3D and 3E. We tested this by measuring the size of mitochondria in PASMCs derived from mice treated with the α-miR29 antagonist or vehicle (Figure 2A and 2B and Figure II in the online-only Data Supplement). miR-29 inhibition increased the long axis of mitochondria (Figures 2C and 7A–7C and Figure II in the online-only Data Supplement).

The electron micrograph, which is a 2-dimensional section through a 3-dimensional object, probably understates the resolution of mitochondrial hyperfissioning. If the mitochondria are randomly oriented, we might expect for some to appear nearly circular (>175 nmol/L in both dimensions) and some to have great length. Figure 7B and 7C provides visual examples of vehicle- or α-miR29–treated PASMCs, with mitochondrial long axes marked by arrows. Counts of mitochondria that appear more than twice as long as they are wide (long axis >400 nmol/L) are low in vehicle-treated PASMCs (8 of 160, 5%) compared with those treated with α-miR29 (56 of 177, 32%). This is a 6-fold increase (P < 0.0001 by Pearson χ²). Variation in mitochondrial size was more than doubled, with standard deviation from the median of 104 nmol/L in vehicle-treated compared with 230 nmol/L in α-miR29–treated PASMCs (P < 0.0001 by Brown-Forsythe; Figures 2D, 2D, 7B and 7C and Figure II in the online-only Data Supplement).

We further assessed mitochondrial size using endothelial-like cells derived from human subjects. Specifically, we used induced pluripotent stem cells derived from a healthy control subject and from a patient with BMPR2 mutation–associated HPAH to derive endothelial-like cells for mitochondrial evaluation. Overall, mitochondria from healthy control endothelial-like cells were larger and more variable in size than mitochondria from HPAH patient endothelial-like cells. For both groups, treatment with α-miR29 increased mitochondrial size (Figure 7E–7G).

Discussion

In these experiments, we used human lung tissue and transgenic mouse models of mutant Bmpr2 to demonstrate that increased expression of miR-29 family is a shared feature of human and murine-modeled HPAH. We further demonstrate that functional and molecular markers of insulin resistance are abnormal in HPAH and that this effect is amplified by the estrogen metabolite 16αOHE and associated with elevated miR-29. Furthermore, in the setting of 16αOHE exposure, we demonstrate a beneficial effect of miR-29 antagonism in terms of disease penetrance, hemodynamics, and pulmonary histopathology in our murine model. While correcting the PH phenotype, treatment with α-miR29 also improved indexes of insulin resistance and metabolic dysregulation, as well as mitochondrial morphology. In summary, these findings support the concept that 16αOHE contributes to HPAH pathogenesis in the setting of a BMPR2 mutation and suggest that miR-29 antagonism may provide a novel therapeutic approach for HPAH.

Among the many perplexing features of human PAH, including HPAH, are the cell-level markers of altered energy metabolism, the systemic features of female predominance, and the association of PAH with insulin resistance and the metabolic syndrome. Independently of PAH, sex hormones are associated with insulin resistance and abnormalities of

Figure 4. Peroxisome proliferator-activated receptor-γ (PPARγ) and CAV1, but not CD36, are direct targets of microRNA-29a (miR-29a) and miR-29c. A, miRNA pull-down assays with subsequent quantitative reverse transcription–polymerase chain reaction (PCR) analysis of the affinity-purified mRNA demonstrate that both the PPARγ and CAV1 genes are bound at the 3’ untranslated region by miR-29a and miR29c but not miR29b. Positive and negative controls were included in the assay. As expected, the elastin (ELN) and ABHD5 genes also are bound (positive controls), whereas G6PC and HPRT are not (negative controls). B, A 1.5% agarose gel loaded with 10 μL real-time PCR product demonstrating gene expression as a function of exposure to miR-29a, miR-29c, or scrambled miRNA duplex. miR-29 family direct targets show clear bands of correct size. Nontarget genes show no bands or smear.
Chen et al

α-OHE Modifies HPAH via miR-29

16α-OHE modifies HPAH via miR-29, which may provide some clues as to how sex and metabolism interface. For example, in women, both subphysiological and supraphysiological estrogen levels associate with insulin resistance and type 2 diabetes mellitus, but in men, the association is strongest with high estrogen and low testosterone levels. However, these findings do not provide the precise mechanistic link between sex hormones, cellular and systemic metabolic abnormalities, and PAH.

MicroRNAs are short, single-stranded, non–protein-coding gene products typically 20 to 22 nucleotides long that posttranscriptionally regulate the expression of target genes through interactions with specific microRNAs. There is certainly a growing recognition of the role that microRNAs play in cardiopulmonary diseases, including PAH. Our finding on tissue microRNA arrays from HPAH patients and Bmpr2 transgenic mice showed a consistent pattern of microRNAs distinct from controls. The patterns were also consistent with previous studies related to insulin resistance and metabolic derangements, including a significant and consistent elevation of miR-29 family expression. Our subsequent studies demonstrated a molecular fingerprint consistent with insulin resistance and metabolic derangements that was attenuated by miR-29 antagonism in concert with hemodynamic improvement and improvement in the physiological HOMA-IR metric of insulin resistance. Although requiring replication, our results suggest that miR-29 contributes to HPAH pathogenesis, at least in the setting of elevated 16αOHE. We previously demonstrated that female and male HPAH patients have an estrogen milieu that...
is skewed toward higher levels of 16αOHE, and we believe that this skewed sex hormone metabolism results in miR29 upregulation in HPAH. 16αOHE binds to the estrogen receptors with high affinity, and we suspect that canonical signaling via estrogen receptor α or estrogen receptor β promotes enhances miR29 expression. Importantly, our findings in the murine model did not differentiate according to sex, although mice of both sexes received exogenous 16αOHE in this study.

Our work has similarities and differences to recent work by Mair and colleagues Mair that demonstrated the beneficial effect of estrogen antagonism in attenuating PH in female murine models. Unlike the present study, that study found that although disease was attenuated in females, there was insufficient effect in the male subgroup. This may be attributable to the naturally lower circulating levels of estrogens in males rather than an inherent inability of males to respond to estrogen antagonism. In our model, mice of both sexes were exogenously exposed to the estrogenic compound 16αOHE. Mair et al also found reduced lung aromatase levels in both cultured PASMCs and the pulmonary artery smooth muscle cell layer in the male compared with females animals. This finding suggests that an organ-specific phenomenon related to local estrogen production and metabolism is also possible. Although we found no difference in murine PASMC mitochondrial size according to sex, association of mitochondrial fragmentation and dysmorphology with local aromatase activity is an area for future study.

We previously demonstrated that 16αOHE not only associates with human female HPAH in BMPR2 mutants but also amplifies HPAH penetrance and phenotype among Bmpr2 transgenic mice of both sexes. Our present study is unique in that we identify a potential molecular mechanism for this effect: miR-29 promotion of metabolic derangements known to associate with HPAH and IPAH, including insulin resistance and alterations in lipid metabolism. Increased ceramide in the lungs suggests impairment of the fatty acid oxidation pathway. Consistent with this, we previously reported that endothelial cells with BMPR2 mutations have reduced expression of genes involved in fatty acid oxidation. MiR-29 appears to be a critical node in the network of factors associated with glucose and lipid homeostasis, with regulatory roles linked to insulin secretion and the regulation of key lipid-metabolizing genes. Our data suggesting a link between the estrogen metabolite 16αOHE and miR-29 are novel in HPAH, although estradiol has previously been

![Figure 6](Image)

Figure 6. Treatment with anti–microRNA-29 (α-miR29) improves metrics of insulin resistance and metabolic dysregulation. A, Weekly injections of α-miR29 for 6 weeks cause significant induction of peroxisome proliferator–activated receptor-γ (PPARγ) and CD36 protein levels in whole mouse lung, rescuing reduced levels in Bmpr2 mutant. Each lane corresponds to protein from 1 mouse; numbers are densitometry normalized to β-act (β-Act). B, α-MiR29 treatment prevents elevation of homeostasis model assessment of insulin resistance (HOMA-IR) seen in vehicle-treated Bmpr2 mutant mice. Each circle corresponds to 1 animal measured; error bars are SEM. *P values are by nonparametric rank-sum test. C, α-MiR29 treatment prevents ceramide accumulation in the pulmonary vasculature.
shown to induce miR-29 expression in a murine model of hepatic injury.84 Our study had several limitations that warrant discussion. Although the subsequent findings presented suggest that elevated miR-29 is a feature of HPAH, the initial discovery of miR-29 elevation was made with a small number of specimens studied with an array platform that could result in false-positive results. In addition, the manner by which 16αOHE increases miR-29 is an opportunity for future study, although previous work suggests that the suppression of the nuclear factor-κB signaling pathway contributes.84 Other mechanisms that promote derangements in energy metabolism, including how glucose flux and insulin resistance influence pulmonary vascular signaling in the

Figure 7. Anti–microRNA-29 (α-miR29) treatment increases average mitochondrial size. A, A distribution of actual mitochondrial sizes in vehicle- or α-miR29–treated cells that is the best fit for the data (Figure III in the online-only Data Supplement) suggests that the modal size of α-miR29–treated animals is 3 times the modal size of vehicle-treated animals. B and C, Examples of pulmonary artery smooth muscle cells (PASMCs) with vehicle or α-miR29 treatment, with mitochondrial long axes marked by arrows. D, Mitochondrial sizes in endothelial-like cells differentiated from induced pluripotent stem (IPS) cells derived from human healthy controls. α-MiR29 treatment increases size, but the effect is not strong. F, Mitochondrial sizes in endothelial-like cells differentiated from IPS derived from a BMPR2 mutant PAH patient. α-MiR29 treatment causes nearly a tripling of the average size of mitochondria. E, Example of IPS-derived endothelial-like cells cultured from the control subject in D. F, Example of IPS-derived endothelial-like cells cultured derived from the BMPR2 mutant HPAH patient. Note in E and F that that mitochondrial size is smaller in HPAH but increases with α-miR29 treatment.
human BMPR2 and mouse Bmpr2 mutant conditions, are not addressed by these studies and are areas for future study. Although our studies suggest a direct effect of miR-29 on PPARγ expression and energy metabolism, we recognize that alternative results from miR-29 activity that may be pathogenic are possible. In addition, it is notable that miR-29 antagonism improved CD36 and GLUT4 levels despite an absence of data to suggest direct binding of miR-29 to the 3′ UTR of those genes. We suspect that this supports the growing body of literature suggestive that PPARγ directly regulates CD36 and GLUT4, but this requires further study.5,8,6 Furthermore, although reduced BMP signaling appears to be a central feature of many PAH forms, the work presented specifically concerns BMPR2-associated HPAH; whether these findings are directly applicable to other forms of PAH needs to be explored. Finally, we recognize that the complexity of sex hormone associations with regard to the heart adaptation to stress is not addressed by these studies; this may be particularly relevant given the growing concern that although women develop PAH more often, they also appear to live longer after diagnosis than men.28,87,88 Finally, our findings require exploration in other animal models representative of PAH.

Conclusions
We demonstrate a potential mechanism by which 16αOHE promotes HPAH, with the finding of elevated miR-29 in human lung tissue from female patients with HPAH. We confirmed the presence of miR-29 family upregulation in a murine model of PAH using an exogenous estrogen metabolite (16αOHE) applied to animals with mutant Bmpr2 expression. Furthermore, the deleterious effects of 16αOHE, including the PAH phenotype, were attenuated by direct antagonism of miR-29. Coincident with phenotypic improvement, molecular and functional indexes of deranged energy metabolism were attenuated by miR-29 antagonism despite continued exposure to 16αOHE. Although further studies of the precise mechanism by which antagonism of miR-29 attenuates the HPAH phenotype and molecular alterations are warranted, novel therapeutic approaches to treat PAH without the complication of direct sex hormone modification may emerge.

Acknowledgments
We thank the subjects and families who graciously contributed to this work.

Sources of Funding
Funding was provided by National Institutes of Health P01 HL 108800, K08 HL 093363, and K23 HL 098743, as well as by the ENTELLIGENCE Young Investigators Award Program. The project was also supported by CTSA award No. UL1TR000445 from the National Center for Advancing Translational Sciences. Its contents are solely the responsibility of the authors and do not necessarily represent official views of the National Center for Advancing Translational Sciences or the National Institutes of Health.

Disclosures
Dr Austin has grant funding from the National Institutes of Health, ENTELLIGENCE Award Program, and the American Thoracic Society. K.E. Womble and E.P. Dawson hold 2 patents related to the isolation of microRNAs (Nos. 8524448 and 8278035; assignee: Bioventures, Inc.; inventors: E.P. Dawson, K.E. Womble). The other authors report no conflicts.

References
28.
27.
26.
25.
23.
21.
18.
19.
17.
16.


Pulmonary arterial hypertension (PAH) is a highly fatal disease of the pulmonary vasculature with multiple risk factors, including the presence of mutations in the bone morphogenetic protein receptor type II gene, female sex, and abnormal sex hormone levels. Estrogens such as the metabolite 16α-hydroxyestron (16αOHE) may contribute to PAH pathogenesis via several mechanisms, including alterations in cellular energy metabolism although the precise mechanisms remain unclear. In this study, we demonstrate that increased expression of the microRNA-29 (miR-29) family is a modifiable feature of bone morphogenetic protein receptor type II–associated heritable PAH (HPAH), which may have implications for pathogenesis and treatment, including a novel link with 16αOHE. First, we identified increased miR-29 in the lung tissue of human and murine-modeled HPAH. 16αOHE amplified murine-modeled HPAH in association with increased miR-29 levels and molecular markers of insulin resistance. Subsequently, in the setting of 16αOHE exposure, miR-29 antagonism reduced disease penetrance, hemodynamics, and pulmonary histopathology in murine-modeled HPAH. Furthermore, while correcting the pulmonary hypertension phenotype, treatment with miR-29 antagonism also improved indexes of insulin resistance and other markers of abnormal cellular energy metabolism. Together with the observation that female sex and female sex hormones associate with HPAH, our findings support the concept that 16αOHE contributes to HPAH pathogenesis in the setting of a bone morphogenetic protein receptor type II mutation, and this may occur via miR-29–promoting defects in cellular energy metabolism. Although further studies are needed, these findings may support novel therapeutic approaches to treat PAH without the complication of direct sex hormone modification.
Estrogen Metabolite 16α-Hydroxyestrone Exacerbates Bone Morphogenetic Protein Receptor Type II–Associated Pulmonary Arterial Hypertension Through MicroRNA-29–Mediated Modulation of Cellular Metabolism


Circulation. 2016;133:82-97; originally published online October 20, 2015;
doi: 10.1161/CIRCULATIONAHA.115.016133
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/133/1/82

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2015/10/20/CIRCULATIONAHA.115.016133.DC1
http://circ.ahajournals.org/content/suppl/2016/12/26/CIRCULATIONAHA.115.016133.DC2

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIAL

miRNA microarray assays: Primers for real-time qPCR

Eln_mus_120F GCGGACTTCTGGTGAGGAGTTC
Eln_mus_269R CTCCAGGACCTGACTCCAAACG
Col1a1_mus_4184F GCGTAGCCTACATGGACCAG
Col1a1_mus_4330R AAGTTCCGGTAGACTCGTG
Mcl1_mus_932F AGCTTCATCGAACCATTAGCAG
Mcl1_mus_1025R CAAACCCCATCCCAGCCTCTTTG
Pparg_mus_1057F mouse AGCCTGCGGAAGCCCTTTGG
Pparg_mus_1197R mouse CAGCAAGCCTGGGCGGTCC
Sirt3_mus_305F mouse TGGTTGAAGCCCACGGGACC
Sirt3_mus_407R mouse GGCAACCTGTCGCCATC
CD36_mus_4F mouse GGCTGTAGCTGGGAAGCC
CD36_mus_98R mouse AGCATGTCTCCGACTGGCATGAG
Ppargc1a_mus_84F GTGTGTCAGAGTGGAGTGGG
Ppargc1a_mus_197R GAGCAGCAGCAGTCTATGTCAC

The gene HPRT was used to normalize expression. Murine HPRT primer sequences:

Hprt_mus_147F TGCTCGAGATGTCATGAAG
Hprt_mus_249R TTTAATGTAATCCAGGTCG
Verification of miR-29 direct targets

The prediction of miRNA targets using bioinformatics algorithms has high false positive and false negative prediction rates. Though false positive prediction can be excluded by experiments, false negative predictions would miss true targets. To further explore miR-29 potential direct targets, we employed a direct affinity purification method using a miRNA pull-down assay followed by specific quantitative PCR using established protocols. In this approach, synthetic miRNA duplexes carrying a biotin group attached to the 3' end of the miRNA sense strand were transfected into murine PVSMCs. This resulted in the sense strand's subsequent incorporation into the miRNA-induced silencing complex (miRISC). After cell lysis, the miRNA-mRNA complexes were captured on streptavidin beads from which the mRNA was purified and analyzed using quantitative RT-PCR analysis of the affinity purified mRNA. The genes PPARγ, CD36, and CAV1 were evaluated as genes of interest suspected to be directly bound by miR-29. Elastin, and ABHD5 are known targets of miR-29 previously demonstrated, and used as positive controls; in contrast, G6PC and HPRT were used as negative controls with regard to miR-29 direct targeting.

Affinity purification of miR-29 direct targets

The sense strand of miRNA was labeled with biotin at 3'UTR end. The sense and the guide strand was synthesized separately and was annealed to form miRNA duplex. Synthesize Biotin-labeled miR-29a, b, c, scrambled microRNA sense strand or un-labeled guide strand as follows:

miR-29a-Bio:
uagcaccaucgaaucgguauu-Biotin

miR-29a:
uaaccgauucagagucggaag

miR-29b-Bio:
uagcaccaauugaaucuguu-Biotin

miR-29b:
cacugauuucagagucgcca

miR-29c-Bio:
uagcaccaauugaaucgguauu-Biotin

miR-29c:
uaaccgauuuucagagucgcca

Scrambled-Bio:
aauuccgauucguuuaccagca-Biotin

Scrambled:
guguauuccagagucuaauag

**Preparation of miRNA duplex and transfection**

The miRNA sense and guide strand were annealed in 10 mM Tris, pH 7.5, 20 mM NaCl. The mixture was incubated for 2 minutes at 95° and then allowed to cool to room temperature on workbench for 2 hours. The miRNA duplex was transfected into murine
pulmonary smooth muscle cells at miRNA concentrations of 50 nM with lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

**Streptavidin purification of miRNA targets**

Streptavidin purification of miRNA targets was performed according to the literature (PMID: 17889804). After purification, RNA was precipitated and dissolved in 20 μl of RNase-free water. All of the RNA was processed to cDNA with QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA).
**Supplemental Figure 1: miR-29 does not bind to the 3’UTR of the CD36 gene.**

miR29 probes produced a product at cycle numbers comparable to that in scrambled probe, and in which melt curves, above, were characteristic of non-specific products. Specific products will have well-defined melt temperatures, as is the case for PPARγ in the figure above, which melts between 81 and 85 degrees C (black lines for triplicate products). By contrast, CD36 had no well defined melt temperature (grey lines), suggesting non-specific product or primer-dimers.

**Supplemental Figure 2: Kidney histology**

Mice exposed to α-miR29 treatment had no evidence of renal fibrosis upon histologic examination. In addition, there were no detectable abnormalities in terms of the renal glomeruli, tubules, or vasculature. Magnification, 20x.

**Supplemental Figure 3: α-miR29 treatment increases average mitochondrial size**

(A) Apparent mitochondrial long axis size is dependent on orientation of the mitochondria with respect to the cutting plane. For instance, a 500 nM mitochondria at a 60° angle to the cutting plane will have an apparent length of only 185 nM. (B) Assuming a random orientation, a 500 nM mitochondria will have a distribution of apparent sizes as depicted. (C) Distribution of measured sizes (symbols) is strong fit to distribution of expected sizes based on actual sizes as depicted in Figure 7A. (D) Measured sizes used to determine measurements of mitochondrial sizes in endothelial-like cells differentiated from iPS cells derived from human healthy controls shown in Figure 7D. (E) Measured
sizes used to determine measurements of mitochondrial sizes in endothelial-like cells differentiated from iPS cells derived from a BMPR2 mutant HPAH shown in Figure 7E.
Supplemental Figure 1

[Graph showing relative intensity against temperature (°C) with curves labeled PPARγ and CD36]
Supplemental Figure 3

A

500 nM

Cutting Plane

185 nM

60°

B

Distribution of Apparent Sizes for a 500 nM Mitochondria with Random Orientation

C

Distribution of Measured Sizes (symbols) Fit to Expected Values for Distributions from Fig 7A(lines)

Vehicle Treated

α-miR29 Treated

D

Distribution of Measured Sizes (symbols) Fit to Expected Values for Distributions from Fig 7D(lines)

Vehicle Treated

α-miR29 Treated

E

Distribution of Measured Sizes (symbols) Fit to Expected Values for Distributions from Fig 7E(lines)

Vehicle Treated

α-miR29 Treated
여성에게 호발하는 유전성 폐동맥고혈압은 miR-29의 발현 증가를 통해 야기된다

한 기훈 교수 서울아산병원 심장내과

초록

배경
폐동맥고혈압(pulmonary arterial hypertension, PAH)은 폐동맥의 증식성 질환이며, 주로 여성에서 발생한다. 에스트로겐의 대사산물인 16α-hydroxyestrone(16αOHE)는 폐동맥의 고혈압성 변화와 PAH와 연관된 세포 에너지대사의 변화에 관여하는 것으로 보인다. 따라서, 16αOHE가 microRNA-29(miR-29) 가계(family)의 발현도 증가(upregulation)를 통해 유전성 PAH(heritable PAH, HPAH)를 축진시키는지, 그리고 miR-29의 저해는 PAH를 호전시킬 수 있는지, Bmpr2(bone morphogenetic protein receptor type II) 돌연변이 쥐를 이용하여 연구하였다.

방법 및 결과
HPAH 환자를 대상으로 인간 폐조직의 microRNA array 프로파일링을 시행하여, miR-29 가계를 포함한 에너지대사와 연관있는 microRNA의 상승을 확인하였다. miR-29의 발현도는 Bmpr2 돌연변이 쥐에서 2배 높게 발현하였으며, 이를 다시 16αOHE에 1.25μg/㎖로 4주간 노출시킨 결과, 4-8배로 증가하였다. Bmpr2 돌연변이 쥐의 조직분석 결과, peroxisome proliferator-activated receptor-γ 및 CD36의 발현이 16αOHE 또는 HPAH의 조직 유래 단백질에 노출시키면 상승되었다. Bmpr2 쥐를 anti-miR-29로 처리(6주간 20mg/kg 주사)하면 혈역학적, 조직학적, 그리고 에너지대사 이상을 방출하는 마커(marker)들의 프로파일이 변형하였다. 그리고 폐동맥 평활근세포를 Bmpr2 돌연변이 쥐에서 분리해보면, 미토콘드리아의 기능이상이 관찰되는데, 이는 anti-miR-29를 과발현시키면 소실·회복된다. HPAH 환자의 유도인공줄기세포(induced pluripotent stem cell)에서 혈관내피세포와 유사한 세포(endothelial-like cell)를 배양하여 분석하여도 결과는 유사하였으며, 이 역시 anti-miR-29 치료에 의해 호전되었다.

결론
16αOHE는 miR-29의 발현증가를 통해 HPAH의 상태로 유도하며, 이는 분자역학적 에너지대사 지표들을 변화시킨다. miR-29의 기능을 차단하면 in vivo 및 in vitro 상태에서의 HPAH 상태를 호전시키므로, 이는 가능성이 있는 신치료기술의 타겟으로 기대된다.