Dynamic Changes in Lung MicroRNA Profiles During the Development of Pulmonary Hypertension due to Chronic Hypoxia and Monocrotaline

Paola Caruso, Margaret R. MacLean, Raya Khanin, John McClure, Elaine Soon, Mark Southgate, Robert A. MacDonald, Jenny A. Greig, Keith E. Robertson, Rachel Masson, Laura Denby, Yvonne Dempsie, Lu Long, Nicholas W. Morrell, Andrew H. Baker

Objective—MicroRNAs (miRNAs) are small noncoding RNAs that have the capacity to control protein production through binding “seed” sequences within a target mRNA. Each miRNA is capable of potentially controlling hundreds of genes. The regulation of miRNAs in the lung during the development of pulmonary arterial hypertension (PAH) is unknown.

Methods and Results—We screened lung miRNA profiles in a longitudinal and crossover design during the development of PAH caused by chronic hypoxia or monocrotaline in rats. We identified reduced expression of Dicer, involved in miRNA processing, during the onset of PAH after hypoxia. MiR-22, miR-30, and let-7f were downregulated, whereas miR-322 and miR-451 were upregulated significantly during the development of PAH in both models. Differences were observed between monocrotaline and chronic hypoxia. For example, miR-21 and let-7a were significantly reduced only in monocrotaline-treated rats. MiRNAs that were significantly regulated were validated by quantitative polymerase chain reaction. By using in vitro studies, we demonstrated that hypoxia and growth factors implicated in PAH induced similar changes in miRNA expression. Furthermore, we confirmed miR-21 downregulation in human lung tissue and serum from patients with idiopathic PAH.

Conclusion—Defined miRNAs are regulated during the development of PAH in rats. Therefore, miRNAs may contribute to the pathogenesis of PAH and represent a novel opportunity for therapeutic intervention. (Arterioscler Thromb Vasc Biol. 2010;30:716-723.)

Key Words: pulmonary hypertension ■ small RNA molecules ■ gene regulation

Pulmonary arterial hypertension (PAH) is a complex disorder characterized by the obstructive remodeling of pulmonary arteries, leading to a progressive elevation in pulmonary arterial pressure (PAP) and subsequent right-sided heart failure and death. Familial PAH is associated in 80% of cases with diverse heterozygous mutations in the gene-encoding bone morphogenetic protein receptor 2 (BMPR-II) and can be associated with mutations in the activin–receptor kinaselike 1 gene. The cause of the variable phenotypic expression of PAH among carriers of mutated BMPR-II genes is unclear, and is likely related to environmental and genetic modifiers. Although BMPR-II-related pathways are considered pivotal, many other mediator pathways participate in the pathogenesis of PAH and are being actively investigated, both independently and in combination. For example, the involvement of serotonin in the development of experimental PAH has been recently reported. Indeed, important interactions between the serotonin and BMP pathways have recently been described. Rats exposed to hypoxia or injected with the toxin monocrotaline develop pulmonary arterial changes correlated with the development of PAH, including remodeling and elevating PAP.

MicroRNAs (miRNAs) are small noncoding transcripts of 16 to 29 nucleotide RNAs that regulate gene expression posttranscriptionally by targeting miRNAs. Animal miRNAs are processed from longer primary transcripts (primary miRNAs) that can contain multiple miRNAs. This precursor is then processed in 3 steps. First, a complex comprising the nuclear RNase III enzyme Drosha and the double strand (ds) RNA-binding protein Pasha/DGCR8 cleaves the primary miRNA in an approximately 60 nucleotides (nt) precursor miRNA hairpin. After this, a shorter precursor is actively transported to the cytoplasm by Exportin-5, where the precursor miRNA undergoes further processing into an approximately 21- to 22-nt duplex (the mature miRNA) by the multidomain ribonuclease Dicer and cofactors. One strand of the duplex is preferentially selected for entry into a silencing complex that includes an argonaute protein. This
mature miRNA guides the argonaute complex to complementary sites on target transcripts.12

Recent studies have measured changes in the synthesis of several thousand mRNAs and proteins in response to miRNA transfection or endogenous miRNA knockdown and have found that the major sequence determinant in the miRNA-mediated downregulation of target miRNAs and proteins is the 6-mer “seed” located in the 3’ untranslated terminal regions (UTRs) of miRNAs.13 Seed sequences in 3’ UTRs were shown to be strongly correlated with reduced protein production, whereas the correlation was weaker but still detectable for seeds in coding sequences.13 The interaction between miRNA and the seed region leads to a blockade of translation via either cleavage of its mRNA and/or translational repression.

Recently, a substantial level of interest in miRNAs has demonstrated their fundamental role in disease pathogenesis. Many recent studies14–16 have reported distinct miRNA signatures in cancer cells. In the heart and vasculature, miRNAs are associated with remodeling and the development of hypertrophy and failure.17–19 In the lung, a recent study20 has highlighted the importance of miRNAs in the regulation of miRNAs after smoke inhalation injury. Herein, we use 2 distinct and commonly used rat models (hypoxic and monocrotaline) of PAH to determine the regulation of miRNAs during disease initiation and progression. We demonstrate time- and insult-dependent changes in miRNA levels that may provide new insights into pathways contributing to the pathobiology of PAH and identify specific miRNAs that are targets for future interventional studies.

Methods
A Supplement (available online at http://atvb.ahajournals.org) provides complete details on the methods used in this study.

Animal Models
Male Sprague-Dawley rats (age, 6 to 7 weeks; weight, 250 to 300 grams) were used throughout. Animals were anesthetized intramuscularly with ketamine, 75 mg/kg, and xylazine, 6 mg/kg; and euthanized at 2, 7, and 21 days after the initiation of hypoxia or after monocrotaline injection. Rats were exsanguinated, and the lungs were removed for analysis.

Two-Channel Microarray Experiment
A 2-channel microarray analysis using the MRA-1003 rat miRNA microarray based on mirBase, version 10.1 (LC Sciences, Houston, Tex) was used. Supplemental Figure IA depicts the design of the miRNA microarray study investigating PAH in rats. To assess the statistical significance of intergroup differences, rank products21 were used. Significance was assessed using the false discovery rate multiple testing correction method,22 with a false discovery rate cutoff of 5%.

Prediction of Potential miRNAs Targets
A list of targets for miR-21, miR-22, miR-30c, miR-451, and miR-322 was obtained by searching 3’ UTR sequences of rat mRNAs for the seeds of miRNAs. Rat mRNAs were downloaded from the UCSC Genome Browser (available online at http://genome.ucsc.edu/). Target miRNA expression was assessed in the same samples used for miRNA analysis using assay kits (TaqMan, Applied Biosystems, Foster City, Calif).

Transforming Growth Factor β1 and BMP4 Rat Pulmonary Artery Endothelial Cells Stimulation
Passage 4 rat pulmonary artery endothelial cells (PASMC) were cultured in 6-well plates in growth media. After 24 hours, the media was replaced with serum-free media and cells were incubated for a further 24 hours. Wells were then incubated with 1 ng/mL of transforming growth factor (TGF) β1 or 10 ng/mL of BMP4 or with Dulbecco-modified Eagle growth media containing 0.1% heat-inactivated fetal calf serum as a negative control. Total RNA was extracted after 5 and 24 hours of stimulation and stored for quantitative polymerase chain reaction (q-PCR) analysis of miR-451, miR-21, miR-22, miR-30c, and let-7f expression.

Results
Time Course of Pulmonary Hemodynamics in Hypoxia-Exposed and Monocrotaline-Treated Rats
Hemodynamic studies confirmed that both chronic hypoxia and monocrotaline exposure resulted in the development of PAH. In control rats, the mean PAP was 20.0 ± 0.8 mm Hg. In hypoxic animals, the mean PAP after 2, 7, and 21 days was 24.8 ± 1.2, 33.9 ± 1.1, and 40.7 ± 1.9 mm Hg, respectively. In monocrotaline-exposed animals at the same time points, the mean PAP was 19.9 ± 0.8, 19.3 ± 1.4, and 29.6 ± 2.1 mm Hg, respectively.

Analysis of Genes Critical for miRNA Biogenesis After the Initiation of PAH
First, we assessed the mRNA levels for genes critical to miRNA biogenesis (ie, Dicer, Drosha, Pasha/DGCR8, and Exportin-5) by TaqMan q-PCR analysis. Interestingly, we observed that in vivo exposure to hypoxia induced a marked and sustained decrease in mRNA for Dicer (Figure 1A and Supplemental Figure IIA). Monocrotaline injury had a significant effect on Dicer expression only 21 days after the treatment (Figure 1A). We also quantified Dicer, Drosha, Pasha/DGCR8, and Exportin-5 mRNA expression in 24-hour hypoxic rat pulmonary artery fibroblasts (PAF), extracting RNA in sextuplicate from cells. In vitro, Dicer expression was significantly downregulated by hypoxia (Figure 1B and Supplemental Figure IIB).

Global miRNA Profiling of the Lungs of Hypoxia- and Monocrotaline-Treated Rats
The expression level of 350 miRNAs in the lung was investigated using a 2-channel microarray experiment, allowing us to identify their expression pattern during the first stages of PAH pathogenesis (Supplemental Figure IB and Supplemental Table I). We confirmed the prominent expression of miR-195 and miR-200c in rat lung samples of control animals (data not shown), consistent with the previous identification of these miRNAs as the only lung-specific miRNAs.23 The expression of miR-322, miR-451, miR-21, miR-22, miR-30c, let-7f, and let-7a was the most significantly altered (Supplemental Table II) and was, therefore, selected for further analysis. Further visualization of miRNA dysregulation was observed using dot plots for average values in each group plotted against each condition and time point (Figure 2).

Validation of the miRNA Dysregulation
To validate the microarray analysis, the expression level of miR-322, miR-451, miR-21, miR-22, miR-30c, let-7f, and let-7a plus a control miRNA that was not altered in the lungs of treated animals compared with controls (miR-145) was analyzed by TaqMan q-PCR using total RNA extracted from both hypoxic and monocrotaline-treated rats. As expected, and in agreement with the profiling data, miR-145 levels were not altered under either condition (Figure 3 and Supplemental Figure III). In
contrast, miR-451, miR-322, let-7f, let-7a, miR-21, miR-22, and miR-30c were significantly altered after injury (Figure 3 and Supplemental Figure III). In particular, miR-451 expression showed a strong upregulation in the hypoxic samples, and reached a maximum 7 days after monocrotaline injection. MiR-322 was significantly upregulated just 7 days after the injury for both the models before returning to control levels by day 21. MiR-22 expression showed differences between the 2 models, resulting in maximal downregulation after 21 days in hypoxic conditions and 2 days after a monocrotaline injection. MiR-21 and miR-30c showed a significant downregulation in the monocrotaline and in the hypoxic model alone, respectively (Figure 3 and Supplemental Figure III). Let-7f expression was downregulated in both the models at early time points in the hypoxic model (2 and 7 days after the induction of hypoxia) and at later time points in the monocrotaline model (21 days after the injection), whereas let-7a showed a significant downregulation only 21 days after monocrotaline injection (Figure 3 and Supplemental Figure III). We also quantified the expression level of the miR-17–miR-92a cluster (miR-17, miR-19b, miR-20a, and miR-92) in consideration of its recent involvement in the regulation of BMPR-II. An array analysis showed no significant dysregulation (Supplemental Table III). MiR-18a and miR-19a were not assessed because they showed low expression on the array. TaqMan q-PCR validations showed a small, but significant, upregulation of miR-17, miR-19b, miR-20a, and miR-92 in the samples (Supplemental Figure IV), consistent with a recent study.24

In Vitro Validation of miRNA Expression Level

Next, we assessed the expression level of miRNAs that were downregulated (miR-22, miR-21, miR-30c, and let-7f) in vitro models of hypoxic injury to determine whether the hypoxic insult in vitro paralleled the effect of miRNA profiles in vivo. We extracted total RNA from normoxic and 24-hour hypoxic rat PAFs and rat PASMCs and analyzed the samples by TaqMan q-PCR for miRNA levels, normalizing this to the internal control U87. The expression pattern for miR-22, miR-21, let-7f, and miR-30c was quite similar to the in vivo analysis. In PAFs, miR-22 and miR-30c showed a significant downregulation, whereas miR-21 and let-7f did not show altered expression after hypoxic treatment (Figure 4B). Thus, the exposure of rat PAFs and PASMCs in vitro to hypoxia correlated well with the changes in miRNAs observed in vivo. We also used 2 in vitro

![Figure 1](http://atvb.ahajournals.org/)

Figure 1. In vivo and in vitro expression level of Dicer, Exportin-5, Drosha, and Pasha in hypoxic and monocrotaline-treated rats. A, q-PCR analysis of hypoxic and monocrotaline-injected rats after 2, 7, and 21 days of exposure. Total RNA was extracted from hypoxic (gray bars) or monocrotaline-injected (black bars) male rats at the age of 6 weeks. Results were normalized to GAPDH values and expressed as relative fold change, with an arbitrary value of 1 assigned to the control (CTR) group. For statistical analysis, we conducted a t test, checking each hypoxic or monocrotaline group vs the CTR group (\(P < 0.05\) and \(**P < 0.001\) vs CTR samples). B, q-PCR analysis of hypoxic rat pulmonary arterial fibroblasts (PAF) showing the expression level of Dicer, Exportin-5 (Exp5), Pasha, and Drosha after 24 hours of exposure. This experiment was performed with total RNA extracted from both the CTR and the hypoxic cells. Results were normalized to GAPDH values (\(***P < 0.001\) vs CTR samples).
human cell models for assessing miRNA modulation. We analyzed human fetal PAFs and human pulmonary artery endothelial cells (PAEC) after 24-hour exposure to hypoxia. The analysis of PAFs showed a significant dysregulation of all 4 miRNAs; in hypoxic PAECs, only miR-30c was modestly downregulated (Supplemental Figure V). Because TGF-β and BMP are important regulators of pulmonary vascular SMC signaling in PAH and the miR-451 promoter contains a Smad-responsive element, we exposed rat PASMCs to TGF-β1 and BMP4 and assessed miR-451 levels. Both agonists induced miR-451 levels significantly after 5 hours of stimulation (Figure 4C). Notably, values returned to the unstimulated level by 24 hours (Figure 4C). For a better understanding of the role of TGF-β1 in the dysregulation of our miRNAs of interest, we also quantified the expression level of miR-21, miR-30, miR-22, and let-7f after stimulation. TGF-β1 stimulation induced the same kind of dysregulation we observed in hypoxic and monocrotaline-treated rats. In fact, all 4 miRNAs (miR-21, miR-22, miR-30c, and let-7f) were downregulated in a time-dependent manner (Figure 4D).

**Prediction of Potential miRNA Targets**

To identify a list of mRNA targets for miR-21, miR-22, and miR-30c, we screened the 3’ UTRs of mRNAs for the 6-mer seeds of the miRNAs of interest. The seed is Watson-Crick consecutive base pairing between mRNAs and the miRNAs at nucleotide positions 2 to 7 from its 5’ end. It has been demonstrated using an unbiased computational approach applied to high-throughput pulsed SILAC data sets that the seed sequence in the 3’ UTR is a primary motif that correlates with both miRNA degradation and translational repression. Seed-based target prediction methods had the highest overlap with pulsed SILAC data. The accuracy of target prediction methods is improved by using evolutionary conservation as an additional filter (PicTar, TargetScanS, or Diana). However, the false-positive rate of these target prediction methods, even with conserved seed incorporation, is still around 40%. The relatively mild extent of miRNA-mediated target downregulation has been found to depend on the number of seeds of the miRNA of interest and the distance between the adjacent seeds. Multiple nonconserved seeds, in particular those located at optimal distances of less than 40 nt, act synergistically. For example, 2 nonconserved seeds located within 40 nt appear to exert the same effect as a single conserved seed (Raya Khanin, PhD, unpublished data, 2009). In addition, seeds for different miRNAs within an optimal distance may cooperatively regulate 1 target. The current target prediction algorithms do not consider this. Therefore, we opted to search for 6-mer seeds for miRNAs of interest in 3’ UTRs of rat mRNAs. In particular, we focused on those proteins that have multiple seeds for 1 or more miRNAs located within a small distance on 3’ UTRs. Based on this, we generated a number of targets for downregulated miRNAs (miR-21, miR-30c, and miR-22) (Supplemental Table IV). The same strategy has been used for preliminary identifi-
cation of the potential targets of the 2 upregulated miRNAs (miR-451 and miR-322) (Supplemental Table V). Several targets identified for the upregulated miRNA group are in common with the group of downregulated miRNAs.

Analysis of miR-21, miR-22, and miR-30c Targets in Hypoxic and Monocrotaline-Treated Lungs

Based on the targets identified in the Supplemental Table IV, we performed TaqMan q-PCR validation analysis on the same sample sets used for miRNA profiling (Supplemental Figure VI). In general, a number of the predicted genes, but not all, were regulated as expected. For example, KCNJ6 mRNA levels were significantly enhanced after both hypoxia- and monocrotaline-induced injuries (Supplemental Figure VI). MiR-21 was only downregulated in monocrotaline-induced injury. Likewise, TACC1, which has predicted targets for miR-21 and miR-30c, was only upregulated in the monocrotaline-treated lungs, suggesting that miR-21 is a key regulator in monocrotaline-induced injuries.
PAH. Protein phosphatase 2, regulatory subunit β’, epsilon isoform (PPP2RSE) was upregulated only after monocrotaline-induced injury, again perhaps indicative of miR-21 regulation. Similarly, transforming growth factor, beta receptor 1 (TGFBR1), a target of miR-22, showed significant upregulation selectively in the monocrotaline model. The upregulation of YWHAZ and TNRC6A only in the monocrotaline-treated rats could be indicative of a significant role of miR-30c in gene regulation after injury (Supplemental Figure VI). To validate the upregulation of targets at the protein level, we quantified by western blot analysis the expression level of TGFBR1 in monocrotaline model 7 days after the treatment (Figure 5B–D).

Expression Level of miR-21 and miR-451 in Samples From Idiopathic Pulmonary Arterial Hypertension in Humans
Considering the high level of dysregulation both in vivo and in vitro of miR-451 and miR-21, and TGF-β1 effect on their expression level, we analyzed total RNA extracted from paraffin-embedded lungs of patients with idiopathic pulmonary arterial hypertension (PAH) and unaffected controls. A significant downregulation of miR-21 was observed (Figure 6A). Similarly, miR-21 downregulation in human samples was also demonstrated in serum samples obtained from patients with idiopathic PAH when compared with serum obtained from unaffected controls (Figure 6B). MiR-451 was unaltered in both lung tissue and serum (Figure 6).

Discussion
Gene regulation by miRNA in the progression of several diseases is becoming increasingly relevant. Herein, we profiled miRNA signatures in the rat lung and compared them longitudinally with rats exposed to either chronic hypoxia or monocrotaline injuries at 2, 7, and 21 days. We show that a small subset of miRNAs is significantly changed (eg, miR-21, miR-22, miR-30c, let-7f, let-7i, let-7a, miR-322, and miR-451). These miRNA profiles were validated by TaqMan q-PCR. The regulation of these miRNAs was also mimicked in vitro models involving exposure of rat PAFs, rat PASMCs, human PAFs, and human PAECs to hypoxia, or exposure of rat PASMCs to TGF-β or BMPs. By using bioinformatics analysis, we also predicted the

Figure 4. miRNA expression is regulated by hypoxia in vitro and TGF-β1-BMP4 stimulation can induce the significant dysregulation of miR-451, miR-21, miR-22, miR-30c, and let-7f in PASMCs. A and B, q-PCR analysis of hypoxic rat pulmonary arterial fibroblasts (PAFs) (A) and PASMCs (B) showing the expression level of miR-21, miR-22, miR-30c, and let-7f after 24 hours of stimulation. These experiments were performed with total RNA extracted from both the control (CTR) and the hypoxic cells. Results were normalized to U87 values and expressed as relative fold change, with an arbitrary value of 1 assigned to the CTR group. *P<0.05, **P<0.005, and ***P<0.001 vs CTR samples.

Figure 5. Assessment of TGFBR1 protein expression level in total rat lung protein extracts and its localization in rat lung paraffin sections. A, TGFBR1 expression level was quantified by Western blot analysis in proteins extracted from lung samples of monocrotaline-treated rats on day 7 (Mday7) and control (CTR) rats. Four samples were tested for each group, and the intensity of the Western blot bands was measured using specific software (Scion Image software). The resulting quantification bars are represented in the graph, with the monocrotaline day 7 group represented by the gray bar (*P<0.05 vs CTR samples). TGFBR1 was localized in lung paraffin sections using an immunohistochemistry assay. B, TGFBR1 staining in CTR rat lungs. C, TGFBR1 staining in lungs after monocrotaline exposure for 7 days. D, Lung stained with a nonimmune isotype-IgG CTR antibody.
target sequences for these miRNAs, which predicted relevance to genes involved in pathways known to be critical in PAH and potentially novel genes. A number of predicted targets were regulated in a manner predicted by the miRNA dysregulation pattern. Moreover, an analysis of targets of the downregulated miRNAs (ie, miR-21, miR-22, and miR-30c) and targets of the upregulated miRNAs (ie, miR-451 and miR-322) showed the presence of several target proteins in common, an indication of the potential complex regulation of such targets in vivo. This could explain why the expression level of several targets did not always correlate with miRNA modulation.

Hypoxia and monocrotaline induced consistent changes in miR-451 and to some extent miR-30c, yet regulated miR-21 and miR-22 differently. This suggests that hypoxia- and monocrotaline-induced PAH share some common elements relating to miRNA regulation and differential regulation. Such differential regulation is not entirely unexpected as the result of the differential pathobiology induced by hypoxia and monocrotaline. Monocrotaline challenge mainly targets the pulmonary vascular endothelium and triggers the inflammatory process, especially monocyte recruitment, both of which play an important role in the course of human PAH.\(^{28-30}\) The course of development of PAH is also different in each model, as is well-known and confirmed in the present study. Hypoxia stimulates changes in medial, adventitial, and endothelial layers of the pulmonary artery; however, hypoxia leads to less inflammation than monocrotaline. The underlying pathobiology in both models leads to differences at the protein level. For example, by using a proteomic approach, it has recently been shown that in the hypoxic model, proteins of the nitric oxide, carbon monoxide, and vascular endothelial growth factor pathways are significantly increased.\(^{31}\) In the monocrotaline model, proteins involved in serotonin synthesis, the enhanced unfolded protein response, and intracellular chloride channels are significantly elevated.\(^{31}\)

Dysregulation of BMP and TGF-β signaling occurs in both the hypoxic and monocrotaline models of PAH. Specifically, reduced lung expression of BMPR-II and reduced signaling via the downstream Smad1/5 pathway are observed.\(^{25}\) Conversely, increased TGF-β signaling is observed, with increased Smad3 phosphorylation and increased expression of TGF-β target genes.\(^{25}\) These effects are greater in the monocrotaline model compared to exposure to chronic hypoxia. Interestingly, the promoter region of miR-451 possesses an Smad3 response element.\(^{26}\) Thus, activation of TGF-β signaling could be responsible for the increased expression of miR-451 observed in our studies. TGF-β stimulation was able to induce the downregulation of miR-21, miR-22, miR-30c, and let-7i in our experiments.

The involvement and importance of miR-21 in disease pathogenesis is becoming increasingly well documented, especially for the cardiovascular system. In blood vessel remodeling after acute vascular injury, miR-21 is rapidly upregulated when it associated with the development of intimal hyperplasia.\(^{32}\) Knockdown of miR-21 blocked development of the lesion, suggesting a critical role for miR-21 in vascular pathology. Herein, we show that miR-21 is selectively and consistently (at all 3 points) downregulated after monocrotaline injection, but not during hypoxic exposure. Therefore, in the lung after exposure to monocrotaline, but not hypoxia, vascular remodeling is associated with a substantial loss of miR-21. Therefore, it will be important in future studies to ascertain the role of miR-21 in monocrotaline-induced pathology. BMPs are known to drive smooth muscle cell differentiation via upregulation of miR-21.\(^{33}\) The downregulation of miR-21 in the monocrotaline model, possibly related to reduced BMP signaling,\(^{35}\) may be involved in the alteration of the smooth muscle cell phenotype that is seen in PAH. Similarly, for other modulated miRNAs, it will be critical to ascertain their role in disease pathology and to define the potential effect of miRNA modulation in disease prevention. In the future, it will be important to generate reliable methods to deliver miRNA therapeutics to the lung efficiently and selectively. This might require new strategies to modulate the tropism of a gene delivery system (eg, viruses) and to generate selective agents and cells.

We have focused on those miRNAs showing the largest changes after the onset of injury. Three important recent studies have shown that even subtle changes in miRNA lead to large changes in phenotypes.\(^{13,27,34}\) Thus, the large changes we have observed in miRNAs in this study are likely to strongly affect the cell biology and pathobiology associated with the development of PAH. The downregulation of miR-21 in idiopathic PAH human samples suggests a potential important role for this
miRNA in human PAH. On the contrary, we did not observe miR-451 upregulation in the same samples.

In summary, for the first time to our knowledge, we have reported the global miRNA profiles of rat lungs after exposure to hypoxia and monocrotaline injury. This highlights the potential importance of miRNA in disease progression and possible targets (either the miRNAs themselves or their mRNA targets) for future therapeutic intervention.

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Disclosures
None.

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8. Caruso et al miRNAs in Pulmonary Hypertension
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Supplement Material

Supplement Methods

TaqMan q-PCR Analysis of Mature miRNAs and mRNAs

For the quantitative PCR (q-PCR), reactions were incubated in a 386-well optical plate at 95°C for 10 min, following by 40 cycles of 95°C for 15 s and 60°C for 1 min. Results were normalized to U87 or Rnu-48 values for miRNAs and to GAPDH for gene expression. The fold change for every miRNA or mRNA expression was obtained. The q-PCRs for each miRNA or mRNA were run in triplicate and results are presented as the mean ± standard deviation of samples. To assess the statistical significance of intergroup differences, a Student’s t test was performed.

MiRNA extraction from frozen lungs, PASMCs, PAFs, PAECs and human serum and reverse transcription

Total RNA from tissues and cells were obtained using the miRNeasy kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions, treated with the DNAse 1, amplification grade (Sigma, St. Louis, MO, USA), to eliminate the genome DNA contamination and quantified using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Absorbance of the RNA samples was quantified at 260 and 280 nm, and the 260/280 ratio was calculated. The samples showed a 260/280 ratio ≥ 1.9, which was assumed as an indicator of RNA purity. cDNA was synthesized from total RNA using stem-loop reverse transcription primers according to the TaqMan MicroRNA Assay protocol (Applied Biosystems, Foster city, CA, USA). Each reaction contained 50 ng of extracted total RNA, 50 nM stem-looped RT primer, 1 × RT buffer, 0.25 mM each of dNTPs, 3.33 U/µl Multiscribe reverse transcriptase and
0.25 U/µl RNase inhibitor. The 15 ml reactions were incubated in a 96-well plate for 30 min at 16 C, 30 min at 42 C, 5 min at 85 C and then held at 4 C.

miRNAs extraction from paraffin-embedded human lungs

The RecoverAll total RNA Isolation kit (Ambion, Streetsville, Canada) was used to extract total RNA (including miRNA) from FFPE samples. Three 10 μm slices were deparaffined with xylene for 3 min at 50°C, washed twice with ethanol, and digested with protease at 50°C for 15 min, then for 15 min at 80°C. The lysate was passed through a filter cartridge and DNAse digested, then RNA was eluted in 30 µl of RNAse free water and quantified using the NanoDrop ND-1000 Spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA).

Primary Culture of Rat Pulmonary Artery Fibroblasts (PAFs)

Male Wistar rats (specific pathogen free, Harlan UK Ltd, 6 weeks old; 300-400g) were used throughout. Fibroblasts were prepared using the technique of Freshney.

Primary Culture of Rat Pulmonary Artery Smooth Muscle Cells (PASMCs)

Rat PASMCs were derived from small precapillary pulmonary arterioles using an iron oxide magnetic separation method, as previously described.

Primary Culture of Human Pulmonary Endothelial Cells (PAECs)

Human Pulmonary Endothelial Cells were obtained from Lonza (Lonza Group Ltd, Basel, Switzerland) and cultured by following the Supplier’s instructions.
Growth of Cells in a Hypoxic Environment

A humidified temperature-controlled incubator (Galaxy R model, Wolf Laboratories, UK) was used as a hypoxic chamber. This incubator allows control of internal oxygen levels between 0 and 21% while CO₂ level is simultaneously controlled at 5%.

Western Blot Analysis

Rat lung tissues removed from control and 7 day monocrotaline-treated male Sprague-Dawley rats were homogenated in lysis buffer containing 150 mM NaCl, 20 mM Tris HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X 100, 2.5 mM napyrophosphate, 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM Na₃VO₄, 1 mM βglycerophosphate and 1 µg/ml leupeptin. The homogenates were sonicated and incubated 20 min on ice. Lysed tissues were centrifuged at 14,000 rpm at 4°C for 15 min. The protein content was determined using a BCA assay (Pierce, Rockford, IL, USA). After blocking in TBS–Tween 0.1%–Milk 10%, filter was incubated with an anti-TGFBR1 antibody (Santa Cruz Biotechnology Inc.) overnight at 4°C. The membrane was then incubated with secondary antibody (dilution 1:1000, goat anti-rabbit Abcam, Cambridge, UK) for 1 hour at room temperature. The ECL Western Blotting detection kit (Amersham, Little Chalfont, UK) was used to detect the presence of the protein of interest on the membrane. Equal sample loading was verified by stripping the blot and reprobing it with an anti-β-actin antibody (Abcam, Cambridge, UK). Protein quantification was performed with the Scion Image software (www.scioncorp.com): band intensities of the protein of interest were established and normalized to the relative β-actin signal.

Immunohistochemistry

Rat lungs were fixed in 4% paraformaldehyde solution at 4°C for 18 hours and embedded in paraffin. After deparaffinization with graded concentrations of xylene and
ethanol, slides were immersed in 3% H$_2$O$_2$ in phosphate buffered saline (PBS) for 30 minutes at room temperature to block endogenous peroxidase activity. Then, they were incubated with 20% normal goat serum for 30 minutes to reduce non-specific background staining. The sections were then incubated with rabbit anti-TGFBR1 antibody (Abcam, Cambridge, UK) at 4 µg/ml or isotype matched rabbit IgG nonimmune control (Dako, High Wycombe, UK). Sections were then incubated with appropriate biotinylated secondary antibody (Dako, High Wycombe, UK) diluted 1:200 in 1% (w/v) BSA in PBS, and then horseradish peroxidase-labelled Extravidin™ (Sigma, St. Louis, MO, USA) diluted 1:400 in 1% (w/v) BSA in PBS. Colour was developed using 3.3 diaminobenzidine-nickel and the nuclei were counterstained with Mayer’s haematoxylin.

**Statements**

Animal procedures were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 (Home Office license PPL60/3773) and with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

**References**

Supplement Figure Legends

Supplement Figure I. Global miRNA profiling. (A) Design of the miRNA microarray study investigating PAH in hypoxic or monocrotaline-exposed rats. Each circle represents animal groups (minimum n = 5/group), with each hypoxia treatment being in red and each drug treatment in blue. The control group is represented in green. Each arrow represents a two-channel microarray experiment. (B) Heat map showing the expression level of all miRNA in the global profiling protocol. Refer to Supplement Table 1 for complete microarray dataset.

Supplement Figure II. q-PCR curves showing DICER and DROSHA expression level in the lung of hypoxic or monocrotaline-injected six weeks male rats. (A) *In vivo* q-PCR result showing the dysregulation of both DICER and DROSHA after a hypoxic/monocrotaline treatment. For every gene, the time point with the maximum dysregulation for both the models has been chosen and the results are showed in duplicate. (B) *In vitro* q-PCR result showing DICER downregulation in 24 hours hypoxic rat PAFs versus untreated cells. The q-PCR curves are shown in duplicate.

Supplement Figure III. q-PCR curves showing miRNA expression levels in the lung of hypoxic or monocrotaline-injected six weeks male rats. For every miRNA, the time point with the maximum dysregulation for both the models has been chosen and the results are showed in triplicate. One example of amplification curve has been chosen for miR-145, taken as negative control.

Supplement Figure IV. Validation of miR-17/92 cluster in hypoxic and monocrotaline rats. q-PCR analysis of hypoxic and monocrotaline-injected rats after 2, 7 and 21 days of exposition. Total RNA was extracted in quadruplicate from hypoxic (grey bars) or monocrotaline-injected (black bars) six weeks male rats. Time
points and samples were tested in triplicate. Results were normalized to U87 values and expressed as relative fold change, with an arbitrary value of 1 assigned to the control group. (*p<0.05, **p<0.005, ***p<0.001 vs control samples).

**Supplement Figure V.** miRNA expression is regulated by hypoxia *in vitro* in human cells. (A and B) q-PCR analysis of hypoxic fetal human PAFs (A) and human PAECs (B) showing the expression level of miR-21, miR-22, miR-30c and let-7f after 24 hours of stimulation. These experiments were performed with total RNA extracted from both the control and the hypoxic cells. Results were normalized to Rnu-48 values and expressed as relative fold change, with an arbitrary value of 1 assigned to the control group (*p<0.05, ***p<0.001 vs control samples).

**Supplement Figure VI: Assessment of miRNA target mRNA levels in rat lung following hypoxic and monocrotaline injuries.** Total RNA was extracted from lung samples of hypoxic (grey bars) and monocrotaline-treated (black bars) rats. cDNA was prepared and mRNA levels were assessed by TaqMan analysis and plotted as fold change vs control samples. Results were normalized to GAPDH values. The miRNAs that possess predicted seed sequences for each target are indicated. (*p<0.05, **p<0.005, ***p<0.001 vs control samples).

**Supplement Table I.** Absolute levels of miRNAs modulated during the progression of PAH. Values are means and standard deviations for miRNA of the intensities for each condition (at least n=5/group).

**Supplement Table II.** Absolute levels of selected miRNAs. Raw values for miRNAs up- and down-regulated in PAH rats are shown.
Supplement Table III. Absolute levels of miR-17/92 cluster. Raw values for miR-17/92 cluster in PAH rats are shown.

Supplement Table IV. Predicted targets for the three downregulated miRNAs miR-21, miR-22 and miR-30c. A list of potential targets for miR-21, miR-22 and miR-30c, obtained through bioinformatic tools, is shown.

Supplement Table V. Predicted targets for the two up-regulated miRNAs miR-451 and miR-322. A list of potential targets in common between the two miRNAs, obtained through bioinformatic tools is shown. Proteins marked in red have been show to be targets also of the down-regulated miRNA group.
Supplement Figure I
Supplement Figure III
Supplement Figure IV

miR-92

Fold Change

CTR day 2 day 7 day 21 day 2 day 7 day 21

Hypoxic
Monocrotaline

Fold Change

miR-19b

Fold Change

CTR day 2 day 7 day 21 day 2 day 7 day 21

Hypoxic
Monocrotaline

Fold Change

miR-17

Fold Change

CTR day 2 day 7 day 21 day 2 day 7 day 21

Hypoxic
Monocrotaline

Fold Change

miR-20a

Fold Change

CTR day 2 day 7 day 21 day 2 day 7 day 21

Hypoxic
Monocrotaline
Supplement Figure V

A

Fold Change

CTR  miR-21  miR-22  miR-30c  let-7f

B

Fold Change

CTR  miR-21  miR-22  miR-30c  let-7f

Supplement Figure V
### Supplement Table I. Absolute levels of miRNAs modulated during the progression of PAH

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Supplement Table IV: Predicted targets for the three downregulated miRNAs miR-21, miR-22 and miR-30c

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<td>KCNJ6</td>
<td>miR-22 and miR-30c</td>
<td>potassium inwardly-rectifying channel, subfamily J, member 6</td>
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<td>PPP2R5E</td>
<td>miR-21 and miR-22</td>
<td>protein phosphatase 2, regulatory subunit B(B56), epsilon isoform</td>
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<td>NFAT5</td>
<td>miR-22 and miR-30c</td>
<td>nuclear factor of activated T-cells 5</td>
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<td>TACC1</td>
<td>miR-21 and miR-30c</td>
<td>transforming, acidic coiled-coil containing protein 1</td>
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<td>ADARB1</td>
<td>miR-22</td>
<td>adenosine deaminase, RNA-specific, B1</td>
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<td>TGFB1</td>
<td>miR-22</td>
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<td>TGFBAP1</td>
<td>miR-22</td>
<td>transforming growth factor, beta receptor associated protein 1</td>
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<td>RBPSUH</td>
<td>miR-21</td>
<td>recombining binding protein suppressor of hairless (Drosophila)</td>
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<td>miR-30c</td>
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<td>YWHAZ</td>
<td>miR-30c</td>
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**Supplement Table V. Predicted targets for the two up-regulated miRNAs miR-451 and miR-322**

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<td>RCAN2</td>
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