Discordant Regulation of microRNA Between Multiple Experimental Models and Human Pulmonary Hypertension

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BACKGROUND: The dysregulation of microRNA (miRNA) is known to contribute to the pathobiology of pulmonary arterial hypertension (PAH). However, the relationships between changes in tissue and circulating miRNA levels associated with different animal models and human pulmonary hypertension (PH) have not been defined.

METHODS: A set of miRNAs that have been causally implicated in PH, including miR-17, -21, -130b, -145, -204, -424, and -503, were measured by reverse transcription-quantitative polymerase chain reaction in the plasma, lung, and right ventricle of three of the most common rodent models of PH: the rat monocrotaline and SU5416 plus chronic hypoxia (SuHx) models and the mouse chronic hypoxia model. Plasma miRNA levels were also evaluated in a cohort of patients with PAH and healthy subjects.

RESULTS: Several miRNA showed PH model-dependent perturbations in plasma and tissue levels; however, none of these were conserved across all three experimental models. Principle component analysis of miR expression changes in plasma revealed distinct clustering between rodent models, and SuHx-triggered PH showed the greatest similarity to human PAH. Changes in the plasma levels of several miRNA also correlated with changes in tissue expression. In particular, miR-424 was concordantly increased (1.3- to 1.5-fold, \( P < .05 \)) in the plasma, lung, and right ventricle of hypoxic mice and in the plasma of patients with PAH.

CONCLUSIONS: miRNAs with established etiologic roles in PH showed context-dependent changes in tissue and circulating levels, which were not consistent across rodent models and human PAH. This suggests different miRNA-dependent mechanisms may contribute to experimental and clinical PH, complicating potential diagnostic and therapeutic applications amenable to these miRNAs.
microRNAs (miRNAs) are short (approximately 22 nt) non-protein-coding RNA molecules that regulate gene expression at the posttranscriptional level by directing the translational inhibition or degradation of cognate messenger RNAs. To date, >2,500 mature human miRNAs have been reported, which are predicted to control the majority of protein-coding genes. The dysregulated cellular expression of miRNAs has been implicated in the etiology of many diseases, including, more recently, pulmonary arterial hypertension (PAH). This progressive disease is characterized by changes in the structure and function of the pulmonary vasculature, which precipitates right-sided heart failure.

A number of miRNAs, including miR-17, miR-21, miR-130, miR-145, miR-204, miR-424, and miR-503, have been demonstrated to be causally involved in the development of pulmonary hypertension (PH). The aberrant expression of these miRNAs in lung vascular cells is known to contribute to the dysregulation in proliferation, apoptosis, and inflammation that underlie PH. Moreover, these miRNAs have been proposed as potential targets for therapeutic intervention. Indeed, strategies designed to reverse pathologic changes in lung miRNA expression have previously shown promise in attenuating hemodynamic and vascular remodeling abnormalities in preclinical animal models of PH.

Although experimental PH induced by the combination of SU5416 and chronic hypoxia (SuHx) in rats has been suggested to most closely model human PAH, miRNAs have mainly been assessed in the monocrotaline (MCT) or chronic hypoxia (Hx) models. Although these models can reproduce important features of human PAH, they generally lack the severe pulmonary arteriopathy observed in patients with PAH. Whether the regulation of miRNA is conserved between these different experimental models has not yet been established, but this may provide important clues to help differentiate model-specific alterations from changes that may relate to more central mechanisms in the pathobiology of PH.

We analyzed the expression of these seven miRNAs across three of the most commonly used rodent models of PH, including MCT rats, SuHx rats, and Hx mice. The expression patterns were assessed in the lung as well as two other biologic sources that have largely been overlooked in previous studies, the right ventricle and plasma. Of note, plasma has become increasingly recognized as a key source of extracellular miRNAs, which have the potential to serve as practical noninvasive biomarkers of disease. This application would be particularly useful in PAH given the difficulty associated with the early diagnosis of this condition. Moreover, the convenience of plasma collection circumvents some of the inherent challenges of obtaining clinically relevant biologic specimens from patients, which can facilitate broader studies on the pathobiology of PAH and provide direct insight into the human disease. We, therefore, also examined the expression pattern of these seven miRNAs in a cohort of patients with PAH and healthy subjects.

We now report a surprising lack of concordance in the pattern of miRNA expression between different rodent models of PH and between experimental and human PH. These findings have important implications for the potential usefulness of miRNAs identified in experimental PH for application as biomarkers and therapeutic targets in human PAH. In addition, our results provide new molecular evidence supporting SuHx-induced PH as a more relevant model of human PAH.

**Material and Methods**

Peripheral blood samples from patients with PAH and healthy subjects were obtained with informed consent, under a protocol approved by the Ottawa Hospital Research Ethics Board (#2011470-01H), as previously described. All animal procedures were approved by the University of Ottawa’s Animal Care Ethics Committee and complied with the principles and guidelines of the Canadian Council on Animal Care. A detailed description of the inclusion/exclusion criteria for human subjects, animal models, plasma isolation, total RNA extraction, RNA quality control assessment, reverse transcription-quantitative polymerase chain reaction, reference control characterization (e-Fig 1), and statistics is provided in e-Appendix 1.

**Results**

**Basal miRNA Expression Patterns Largely Conserved Between Species**

Plasma levels of individual miRNA varied by nearly three orders of magnitude and generally followed the rank order: miR-204 < miR-503 < miR-130b < miR-424 < miR-17 < miR-145 < miR-21 (Fig 1A). Interestingly, this pattern was largely conserved across humans, rats, and mice under physiologic conditions, with the exception of miR-145, which ranked below miR-424 in human plasma (Fig 1A). miRNA expression patterns in the lung and right ventricle were also comparable between rats and mice (Fig 1A), and only modest differences in
the rank order of miRNA expression were evident between plasma and tissues. Overall, a strong positive correlation was observed between plasma miRNA levels and their corresponding expression levels within the lung (Pearson $r = 0.89-0.90$, $P < .01$ for rats; and Pearson $r = 0.95$, $P < .01$ for mice) and right ventricle (Pearson $r = 0.87-0.88$, $P < .05$ for rats; and Pearson $r = 0.80$, $P < .05$ for mice) (Fig 1B). This correlation was consistently observed across rats (both Fischer and Sprague-Dawley strains) and mice.

**Predominant miRNA Downregulation in MCT Rats**

Rats received a single intraperitoneal injection of MCT (or vehicle control), and right ventricular systolic pressure (RVSP) and right ventricular hypertrophy (right ventricle to left ventricle plus septum [RV/(LV + S)] mass ratio) were significantly increased 3 weeks post MCT injection (Figs 2A, 2B). The plasma levels of both miR-322 (the rat homolog of human miR-424) and miR-503 were significantly decreased in MCT rats relative to control rats (1.3-fold down, $P < .01$ and 1.8-fold down, $P < .001$, respectively) (Fig 2C), which mimicked a significant decline in lung tissue expression (3.7-fold down, $P < .0001$ and 5.6-fold down, $P < .0001$, respectively). miR-204 was decreased in lung tissue in MCT rats (1.9-fold down, $P < .01$) and showed a trend ($P = .097$) toward lower levels in the plasma. miR-145 was also decreased in the lung (2.0-fold, $P < .0001$) and right ventricle (1.3-fold, $P < .05$) of MCT rats, whereas no change in plasma level was detected. miR-130b was decreased in the lung of MCT rats (1.7-fold down, $P < .01$) but showed a trend ($P = .07$) toward higher levels in plasma. Only miR-21 was increased, showing trends toward higher levels in...
plasma ($P = .07$) and a significant increase in lung tissue expression (3.2-fold up, $P < .0001$). The plasma, lung, and right ventricle levels of miR-17 were not altered in MCT rats.

**Modest miRNA Changes in SU5416/Hx Rats**

Rats received a single subcutaneous injection of the vascular endothelial growth factor receptor-2 inhibitor, SU5416 (or vehicle control) and were exposed to hypoxic conditions (10% oxygen) for a period of 3 weeks, followed by 5 weeks of normoxic conditions (Fig 3A). Plasma, lung, and right ventricle miRNA levels were measured after 8 weeks, at which time there was a significant increase in RVSP and RV/(LV + S) mass ratio (Fig 3B). Surprisingly, despite the severity of the PH phenotype, levels of these specific miRNAs were not significantly altered in plasma or lung between SuHx and control groups (Fig 3C), and only miR-145 showed a trend toward increased levels in the lung. The only significant changes in tissue miR expression were seen in the right ventricle of SuHx rats, with a twofold increase in miR-21 ($P < .01$) and twofold decrease in miR-204 ($P < .05$) (Fig 3C).

**miRNA Upregulation in Hx Mice**

Mice were exposed to Hx for 3 weeks (Fig 4A), after which time there were modest increases in RVSP and RV/(LV + S) mass ratio (Fig 4B). In contrast to the MCT model, a general pattern of upregulation was observed among the miRNAs that were altered in hypoxic mice. One miRNA in particular, miR-322 (the mouse/rat homolog of human miR-424), was shown to be significantly and concordantly higher in the plasma ($1.3$-fold, $P < .05$), lung (1.4-fold, $P < .001$), and right ventricle (1.4-fold, $P < .05$) of hypoxic mice vs control mice (Fig 4C). The expression of miR-503 was also significantly higher in the lung and right ventricle of Hx mice, but plasma levels were not significantly altered. The plasma levels of miR-17 were significantly elevated in Hx mice (1.4-fold, $P < .01$), whereas no changes in expression were observed in the lung or right ventricle. A trend ($P < .1$) toward higher plasma levels of miR-204 was
evident in Hx mice, but no significant difference in lung or right ventricle tissue was detected. Both miR-21 and miR-145 were increased in the lungs of Hx mice (1.6- and 1.4-fold, respectively; \( P < .001 \)), with no change in plasma or right ventricle levels. No significant changes in the plasma, lung, or right ventricle levels of miR-130b were observed.

**Principle Component Analysis Underscores Differences Between Models**

To gain further molecular insight into the relative similarity or dissimilarity between experimental models, principle component analysis (PCA) was conducted on miRNA expression changes in plasma, lung, and right ventricle. The 21-dimensional miRNA expression space (defined by changes in the seven target miRNAs across three different biologic sources) was linearly transformed and mapped into just two dimensions (ie, principle components) to facilitate visualization and comparison of the variability among individual rodents within and between each PH model (Fig 5A). The distance between any pair of individuals in the PCA plot is related to the degree of similarity between the two individuals in high-dimensional space. The animals in each experimental model formed distinct clusters, with some overlap observed among rodents exposed to hypoxia alone or in combination with SU5416. Animals in the MCT and hypoxia models showed the greatest separation in principle component space, which contrasted with their relative closer proximity in a biplot comparison of their phenotypic abnormalities in RVSP and RV/(LV + S) mass ratio (Fig 5B).

To gain insight into whether the discordance in miRNA regulation between models was also reflected in the regulation of downstream messenger RNA targets, we examined two specific messenger RNAs (mRNAs) (Fgf2 and Rhob) that are known to be direct targets of miR-424/503 and miR-21, respectively (e-Fig 2). Fgf2 transcript levels were significantly elevated in lung tissue from both rat models of PH (1.4-fold in SuHx and 1.5-fold in MCT, \( P < .05 \)), but no significant change was observed in the lungs of hypoxic mice vs control mice. The mRNA level of Rhob was downregulated in SuHx rat lungs (1.2-fold, \( P < .05 \)), whereas no significant change was observed in the lungs of MCT rats or Hx mice. Of note, the increased Fgf2 level in the lungs of...
MCT rats was consistent with the observed decrease in miR-322/503 in this model. However, this expected inverse relationship between mRNA target and miRNA was not directly observed in other models or between Rhob and miR-21 (e-Fig 2).

PCA Exposes Relative Similarity Between Human and SuHx-Triggered PH

To gain insight into which experimental trigger of PH most closely models the molecular changes associated with human PAH, target miRNAs were also measured in plasma from 14 patients with PAH (58 ± 10 years, 57% women) and 13 healthy control subjects (45 ± 13 years, 69% women) (Fig 6A). The PAH patient cohort was composed of both idiopathic (43%) and associated forms (57%) of the disease, of which the majority (79%) were on PH-specific therapy at the time of blood sampling. Detailed clinical characteristics of this patient cohort have been described previously (e-Table 1). The plasma level of miR-17 was decreased in patients with PAH (1.4-fold, \( P < .05 \)), whereas levels of miR-21, -130b, -204, and -503 were not significantly different between patients with PAH and healthy control subjects. However, it should be noted that these miRs were originally linked to PH by interrogation of other biologic samples, including cell and lung tissue specimens, or by comprehensive in silico network analyses. PCA of the plasma changes in these seven miRNAs exposed a relative similarity in the underlying miR regulation between patients with PAH and rodents with SuHx-triggered PH (Fig 6B). In principle component space, SuHx rats showed the closest overlap with patients with PAH and also exhibited a similar degree of heterogeneity in the miRNA response among individuals. In comparison, rodents from the MCT and Hx models were distributed in tighter clusters within the PCA plot.

Potential Diagnostic Biomarkers of PAH

For miRNAs that were altered in the plasma of patients with PAH, receiver operating characteristic (ROC) curve analysis was conducted to gauge their potential
diagnostic usefulness. miR-424 and miR-17 both exhibited significant areas under their corresponding ROC curves (area under the ROC curve of 0.76 and 0.73, respectively; \( P < .05 \)) (Fig 6C) and exhibited the capacity for high sensitivity (0.93 for both miR-424 and miR-17; 95% CI, 0.66–1.00) and moderate specificity (0.69; 95% CI, 0.39–0.91 and 0.62; 95% CI, 0.32–0.86, respectively) in discriminating between patients with PAH and healthy participants. However, no significant correlation was observed between plasma miRNA level and various physiologic indexes of disease severity in this small cohort, including mean pulmonary artery pressure, pulmonary vascular resistance index, and the 6-min walk distance test (data not shown).

Discussion

This study provides one of the most extensive assessments of PH-induced changes in miRNA expression reported to date. The expression patterns of seven miRNAs were analyzed in the plasma, lung, and right ventricle of three of the most commonly used rodent models of PH and in plasma from patients with PAH. We focused specifically on a set of miRNAs that have previously been implicated causally in PAH, based on supporting evidence that included loss- and/or gain-of-function experiments in cells and/or specific animal models.\(^5\)–\(^{12}\) In contrast to previous studies that have investigated the specific molecular mechanisms of individual miRNAs, the current study now helps to answer broader questions about the nature of their regulation across different biologic sources, species, and experimental vs clinical forms of PH. This may provide new perspectives on the molecular pathobiology of PH and important insight on the translational potential of animal studies and the related merits of therapeutic strategies aimed at reversing pathologic changes in miRNA expression.

We found that each of the target miRNAs was detectable in the plasma of mice, rats, and humans. Moreover, the relative rank order of plasma miR levels was highly conserved across species, suggesting that similar mechanisms may control the basal release and clearance of these miRNAs from circulation across these species. Of note, the plasma miR levels in three separate animal models consistently showed strong positive correlations with miRNA levels in the lung. Although not definitive evidence, this correlation does suggest that circulating miR levels may be driven at least in part by the magnitude of lung expression, consistent with the fact that the lung represents the largest vascular bed with the greatest opportunity to release or clear miRNAs across the pulmonary circulation.

Interestingly, none of the PH-associated changes in plasma miRNA levels were common between two or more of the experimental models (Table 1). However, these discordant patterns were not limited to circulating miRNAs, as both lung and RV miRNA expression showed distinct model-specific patterns of change. The known mRNA targets of two miRNAs were also examined in the lungs of each model. Although

Figure 5 – A, B. PCA mapping of miRNA expression changes in three rodent models of pulmonary hypertension. A, Hx (○), SuHx (◇), and MCT (△) denote individual animals from each experimental model. For each animal, the change in seven target miRNAs measured across three different biologic sources (plasma, lung, and RV) were mapped to a position in two-dimensional space (ie, PCs) for easy visualization. The distance between any pair of animals in the PCA plot is related to the degree of similarity between the two observations in higher dimensional space. A total of 47.1% of the entire information content of the 21-dimensional miRNA expression space is captured in the two-dimensional PCA plot, with 32.4% and 14.7% of the variability in data explained by PC1 and PC2, respectively. Ellipses denote 1.5 SDs, and line vectors connect to the centroid. B, Biplot showing distribution of individual animals from various pulmonary hypertension models according to their RV/(LV + S) and RVSP. Data are presented as fold change to mean baseline level in control animals. PC = principle component; PCA = principle component analysis. See Figure 1-4 legends for expansion of other abbreviations.
other miRNA targets. This suggests that in these cases miRNA may not be the sole or even the most important mechanism of regulation for these targets. In previous studies, miRNA expression changes associated with experimental PH have commonly been evaluated in lung tissue, and our assessment of miRNA expression changes were generally quite comparable to previous reports (e-Table 2); however, there were a few exceptions that we speculate could be attributed to differences in the experimental time course and severity of PH or possibly other study-specific factors (eg, animal strain) (e-Table 2).

Of note, within each model system we observed similar changes in the PH-associated miRNA levels in plasma and tissues. A general pattern of downregulation was observed among miRNAs that were altered in the MCT model (ie, five of six altered miRs), which contrasted with a general pattern of upregulation among the miRNAs that were altered under hypoxic conditions. The divergence in miR regulation between these models was further supported by PCA, in which rodents with MCT-induced PH clearly formed a distinct cluster separate from rodents with hypoxia-induced PH. It is intriguing that the same miRNAs are differentially regulated in these two models of PH. Certainly, Hx and MCT produce distinct PH phenotypes that likely involve different molecular mechanisms; however, it remains possible that some changes may reflect off-target effects of the specific stimuli used to induce PH. In contrast, we found only modest changes in the mean miRNA levels in the SuHx model of PH, despite far greater abnormalities in hemodynamics and RV remodeling. Nevertheless, the distribution of individual animals/patients revealed in the PCA plot of changes in plasma miRNA suggests this model may more closely resemble human PAH than the MCT and hypoxia models. This is consistent with the similarities in cellular and histologic abnormalities (ie, plexiform lesions) and progressive irreversible phenotype that have previously supported the SuHx model as most relevant to the human disease. 18

miR-424 was the only miRNA to show concordant changes in the plasma of humans and at least one experimental model of PH, Hx. In hypoxic mice, elevated plasma levels of miR-322 (the mouse analog of human miR-424) also mirrored increased expression within the lungs and right ventricle, suggesting that altered plasma levels may be a surrogate marker of disease activity in damaged/remodelled tissues. To our knowledge, the only other miRNA previously reported to be concordantly altered in the plasma of human PAH and a

confirming the expected inverse regulation between miR-322/503 and Fgf2 in the MCT model, there was also evidence of discordance in other models and for
rodent model of PH is miR-26a, which was decreased in
the plasma, lung, and right ventricle of MCT rats, and
in the plasma and cellular buffy coat of patients with
PAH. However, unlike miR-424, it remains to be estab-
lished whether miR-26a is causally involved in the devel-
opment of PH in the MCT model, which is currently an
area of investigation in our laboratory.

PAH is a rare disease with prevalence estimated at only
15 to 26 cases/million, making large cohort studies
inherently challenging. Although the sample size of this
human patient cohort is limited, it nonetheless can pro-
vide some relevant insight into the relative performance
of these miRs as potential biomarkers of PH. Of the
seven miRNAs investigated in this study, only miR-424
and miR-17 exhibited significant differences in plasma
levels between patients with PAH and healthy control
subjects. The plasma levels of both miRNAs showed
significant areas under ROC curves, indicative of a good
balance between sensitivity and specificity in discrimi-
nating patients with PAH from healthy subjects.

This study has some limitations, including the small
sample size and heterogeneity of subjects in the PAH
cohort. The diagnostic usefulness of possible miRNA-
based biomarkers will clearly need to be validated in
larger prospective cohort studies. It is also uncertain
what effect patients with different forms of PAH and
therapy may have on comparisons with the more
well-defined experimental forms of PH induced in the
animal models. Finally, it is important to note that
cell-specific changes in miRNA (or mRNA) expres-
sion, which may underlie disease activity, could be
obscured by the complex cellular background in tissue
measurements.

Conclusions
Despite their previously established roles in the patho-
biology of experimental PH, the majority of miRNAs
investigated in this study showed limited potential as
circulating biomarkers of this disease. In addition, three
separate experimental models of PH showed marked
disparities in their pattern of miRNA expression
changes in tissue and plasma. These differences have
important implications for how preclinical models are
leveraged to gain insight into the molecular mechanisms
of human PH.
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Additional information: The e-Appendix, e-Figures, and e-Tables can be found in the Supplemental Materials section of the online article.

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