Hypoxia-induced Pulmonary Hypertension in Different Mouse Strains: Relation to Transcriptome

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Abstract

Patients with pulmonary arterial hypertension (PAH) can harbor mutations in several genes, most commonly in *BMPR2*. However, disease penetrance in patients with *BMPR2* mutations is low. In addition, most patients do not carry known PAH gene mutations, suggesting that other factors determine susceptibility to PAH. To begin to identify additional genomic factors contributing to PAH pathogenesis, we exposed 32 mouse strains to chronic hypoxia. We found that the PL/J strain has extremely high right ventricular systolic pressure (RVSP; 86.58 mm Hg) but minimal lung remodeling. To identify potential genomic factors contributing to the high RVSP, RNAseq analysis of PL/J lung mRNAs and microRNAs (miRNAs) after hypoxia was performed, and it demonstrated that 4 of 43 upregulated miRNAs in the *Dlk1-Dio3* imprinting region are predicted to target T cell marker mRNAs.

These target mRNAs, as well as the numbers of T cells were downregulated. In addition, C5a and its receptor, C5AR1, were increased. Analysis of Rho-associated protein kinase (Rock) 2 mRNA expression, in the RhoA/Rock pathway, demonstrated a significant increase in PL/J. Inhibition of Rock2 ameliorated a portion of the elevated RVSP. In addition, we identified miR-150-5p as a potential regulator of Rock2 expression. In conclusion, we identified two possible pathways contributing to the hypoxia pulmonary hypertension phenotype of extreme RVSP elevation: aberrant T cell expression driven by hypoxia-induced miRNAs and increased expression of C5a and C5AR1. We suggest that the PL/J mouse will be a good model for seeking mechanism(s) of RVSP elevation in hypoxia-induced PAH.

Keywords: microRNA; immune cells; *Dlk1-Dio3* imprinting region; proinflammatory cytokines

Pulmonary arterial hypertension (PAH) is characterized by elevated pulmonary arterial pressure (>25 mm Hg) that can lead to right heart failure and death when not treated (1–3). Genetic studies have revealed that some patients with PAH carry mutations, most frequently in the bone morphogenetic protein receptor 2 (*BMPR2*) gene (4), although mutations in *ACVRL1*, *ENG*, *SMAD9*, *CAV1*, and *KCNK3* have also been reported (5). More than 80% of patients with familial PAH and 15–25% of

patients with idiopathic PAH have *BMPR2* mutations (5). However, disease penetrance is low in family members carrying *BMPR2* mutations and with no phenotype suggesting that other factors, including genetic/genomic factors, contribute to PAH susceptibility and development. Several PAH animal models have been reported, including rats or mice exposed to either hypoxia/Sugen5416, monocrotaline, or chronic hypoxia (6–10). In addition, *BMPR2* mutant mice develop PAH only

when exposed to further insults/stimuli, with the resulting PAH being relatively mild (11–13). Although some insight has been gained from these animal models, the etiology and pathogenesis of PAH remain poorly understood.

microRNAs (miRNAs) are small molecules that play important roles in gene regulation by base pairing to their target mRNAs at the post-transcriptional level (14). miRNAs are involved in regulating several biological processes, including cell

(Received in original form December 18, 2017; accepted in final form August 21, 2018)

This work was supported by National Institutes of Health grant HL102107 (W.C.N.).

Author Contributions: Conception and design—K.T.I., M.W.P., T.D.L.C., M.K.P., and W.C.N.; analysis and interpretation—K.T.I., P.T.H., M.W.P., N.D., P.A.P., T.D.L.C., M.K.P., and W.C.N. Drafting the manuscript for important intellectual content—K.T.I., M.W.P., N.D., T.D.L.C., M.K.P., and W.C.N.; final approval of the manuscript—M.W.P., T.D.L.C., M.K.P., and W.C.N.

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This article has a data supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

Am J Respir Cell Mol Biol Vol 60, Iss 1, pp 106–116, Jan 2019

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Originally Published in Press as DOI: 10.1165/rcmb.2017-0435OC on August 22, 2018

Internet address: www.atsjournals.org

growth, apoptosis, and hematopoietic lineage differentiation, and miRNAs play key roles in some diseases, such as cancer and cardiovascular and immune disorders (15). For example, in the *Dlk1-Dio3* imprinting region on human chr14, more than 50 miRNAs are encoded, and several have been implicated in human diseases, mostly cancer (16). Studies have indicated that miRNAs are dysregulated in PAH. Reduced concentration of plasma miR-150 is associated with poor survival of patients (17), and miR-145 expression is elevated in the lung of experimental mouse models and patients with PAH (18).

Studies have also reported a connection between PAH and the immune system. Athymic rats lacking T cells have increased susceptibility to PAH (19, 20), and immune reconstituted athymic rats attenuate the development of PAH (21). Immature dendritic cells (DCs) have been shown to accumulate in remodeled pulmonary vessels in both human and rat pulmonary hypertension (PH) (22). However, the mechanism of aberrant expression of immune cells and their physiological relevance in PAH is not fully understood. Complement component 3 (C3) deficiency can attenuate PAH development in mice, suggesting a role for the complement system in PAH development (23).

To identify susceptibility genes for PAH, we performed a survey of 32 mouse strains exposed to chronic hypoxia. We found that the PL/J strain demonstrates extremely high right ventricular (RV) systolic pressure (RVSP) after exposure. We explored potential genomic factors contributing to this high-RVSP phenotype. We propose two possible pathways whereby hypoxia may regulate both T cell development via miRNA expression as well as levels of C5a, its receptor C5AR1, and miR-150-5p, which activate Rho-associated protein kinase (Rock) 2, resulting in high RVSP in PL/J. Some of the results of these studies have been previously reported in abstract form (24–27).

Methods

Additional methods are detailed in the data supplement.

Mice Model and Hypoxic Exposure

Animal studies were reviewed and approved by the Institutional Animal Care and Use

Committee at Cincinnati Children's Research Foundation. Mouse strains used in this study are summarized in Table E1 in the data supplement. Briefly, female mice, 8–10 weeks of age, were placed in a chamber and exposed to normobaric hypoxia (10% O₂) for 1 day, 3 days, and 1, 3, 4, or 5 weeks. Age- and sex-matched normoxic control mice were also used.

Hemodynamic Measurements and Isolation of Tissues

After anesthesia via inhaled isoflurane (2.5%), the right jugular vein was exposed and a SPR-671 pressure transducer catheter (Mikro-Tip 1.4F; Millar Instruments) was inserted into the jugular vein via a small incision and passed into the RV. Lungs and hearts were harvested after pressure measurements were obtained (*see* the data supplement). Mouse lung was inflation fixed with paraformaldehyde and embedded in paraffin as previously described for histologic analysis and vascular morphometry (11, 28).

RNA Extraction and Quantitative Real-Time PCR

Total RNAs, including miRNAs and mRNAs, were isolated from murine lung using the miReasy mini kit (Qiagen). Primers used for miRNA or mRNA amplification are listed in Table E2 or Table E3, respectively. All real-time PCR was performed on an Applied Biosystems 7,300 Real-Time PCR system.

Deep Sequencing Analysis for MiRNAs and mRNAs

Lung total RNA samples (miRNAs and mRNAs) were isolated from PL/J, MRL/MpJ, and FVB/NJ strains exposed to hypoxia or normoxia as described previously here ($n = 3–4$ per group). Library preparation and deep sequencing analysis was performed on an Illumina HiSeq2000 by the Genetic Variation and Gene Discovery Core Facility at Cincinnati Children's Hospital Medical Center.

StrandNGS version 3.0.1 software (Build 232,555; Strand Life Sciences) was used for the miRNA and mRNA data analysis. miRNA and mRNA sequences in the fastq files were aligned with reference sequences (Mouse mm10). After filtering reads by trimming low quality (average base quality below 20), quantification was performed using the DEseq algorithm. False discovery rates

(FDR) were calculated based on the Benjamini Hochberg algorithm for obtaining differentially expressed genes and miRNAs. Fold change (± 1.5) with FDR corrected P value of 0.05 was used as the criterion for selection of differentially expressed genes and miRNAs.

Cell Preparation and Flow Cytometry

Spleens, lung, and alveolar lavage were harvested from MRL/MpJ and PL/J mice strains and FACS analysis was performed, as previously described (29–32).

DC Stimulation with Complement 5a

DCs (CD11c⁺ CD11b⁺) were purified from the lungs of MRL/MpJ and PL/J mice strains and stimulated in the presence and absence of C5a, as described previously (29).

Statistical Analysis

Comparison of two conditions was performed using unpaired Student's t test. Multiple comparisons to control were performed with Dunnett's test using R3.3.1. The comparison of multiple conditions was performed using a one-way ANOVA followed by Holm's test using js-STAR2012. Error bars in the graphs represent the mean ($\pm$ SEM or SD), as described in the figure legends. A P value less than 0.05 was considered statistically significant.

Results

Survey of RVSP in 32 Inbred Mouse Strains Identifies PL/J as a Highly Sensitive Responder and MRL/MpJ and FVB/NJ as Resistant Low Responders

To identify novel genomic factors contributing to the development of PAH, we focused on a mouse strain demonstrating a PAH-like phenotype, including extreme RVSP, limited lung arterial remodeling, and RV hypertrophy (RVH) under hypoxic conditions. Among 32 mouse strains surveyed, PL/J demonstrated the highest mean RVSP (86.58 mm Hg) after chronic hypoxia (Figure 1A and Table E1). PL/J also demonstrated the greatest increase between normoxic and hypoxic RVSP ($\Delta 53.05$ mm Hg) as compared with the average of $\Delta 15.97$ mm Hg for all 32 strains. FVB/NJ and MRL/MpJ strains showed much lower RVSP increases ($\Delta 11.92$ mm Hg and $\Delta 6.91$ mm Hg, respectively). Based on these

Figure 1. Mouse strain survey of right ventricular systolic pressure (RVSP) under hypoxia exposure. (A) Mean RVSP measurements observed in 32 mouse strains exposed to normobaric hypoxia for 4 weeks compared with normoxic controls. A robust increase in RVSP was observed in the PL/J strain after 4 weeks of exposure to hypoxia (black bar). FVB/NJ and MRL/MpJ showed less of an increase in RVSP after 4 weeks of exposure to hypoxia (shown in medium and dark gray bars, respectively). Data are presented as mean $\pm$ SD from 8–16 mice per strain. We phenotyped a larger number of mice in our low- and high-responder strains (FVB/NJ, MRL/MpJ, and PL/J) to solidify the RVSP values. (B) Experimental scheme for time point hypoxia. Three mouse strains were exposed to hypoxia (10% O₂) for periods of 1 day, 3 days, 1 week, 3 weeks, or 4 weeks. Control mice from each strain remained in normoxia (21% O₂). (C) Mean RVSP measurements observed in FVB/NJ, MRL/MpJ, and PL/J strains at each time point (normoxia, 1 d, 3 d, 1 wk, 3 wk, or 4 wk). Data presented as mean $\pm$ SEM (** P < 0.01, *** P < 0.001). We used three to four mice for 1 day, 3 days, 1 week, and 3 weeks. The 4-week time point includes a larger number of mice (n = 18–26).

results, we further analyzed PL/J as a “highly sensitive responder” strain and FVB/NJ and MRL/MpJ as “resistant low responder” strains to chronic hypoxia. We also characterized the animals for other hallmarks of PH: RVH as measured by

RV / LV + S (where LV is left ventricle, and S is septum) and lung arterial remodeling. Among the 32 strains surveyed, PL/J had the highest RV / LV + S weight ratio (mean $\pm$ SD; 0.44 ± 0.04), whereas FVB/NJ and MRL/MpJ showed much less RVH

(0.30 ± 0.06 , 0.26 ± 0.04 , respectively) after 4 weeks of hypoxic exposure (Figure E1 and Table E1). The percentage arterial muscularization was increased to approximately the same degree in both PL/J and MRL/MpJ after hypoxia compared

with normoxic controls: 19.2% ($P < 0.05$) and 18.0% ($P < 0.01$), respectively. The percentage of wall thickness for both PL/J and MRL/MpJ was increased to approximately the same extent (1.7% and 1.4%, respectively), but not with statistical significance (Figures E2A–E2C). To identify both acute and chronic changes in RVSP, we exposed the mice to varying lengths of hypoxia as well as normoxia (Figure 1B). Although RVSP tended to increase in PL/J animals with acute hypoxia exposure, RVSP was not changed significantly until 4 weeks of hypoxia exposure in FVB/NJ and MRL/MpJ (Figure 1C).

Comparison between PL/J, FVB/NJ, and MRL/MpJ of Top Regulated Genes after Hypoxia Exposure

We first analyzed the overlap of top regulated gene candidates (fold change ± 1.5 with FDR corrected P value of 0.05) among the three different strains: PL/J, MRL/MpJ, and FVB/NJ. We extracted the top 50 upregulated or downregulated mRNAs in each line after 3 weeks hypoxia and created a Venn diagram (Figure 2 and Tables E4 and E5). For upregulated mRNAs, 16 genes are common in the 3 strains. Seven, seven, or three genes are common between PL/J and MRL/MpJ, PL/J and FVB/NJ, or MRL/MpJ and FVB/NJ, respectively. There are uniquely regulated genes for PL/J, MRL/MpJ, or FVB/NJ, which number 20, 24, or 24, respectively. For downregulated mRNAs, 14 genes are shared between the three strains. Three, five, or three genes are common between PL/J and MRL/MpJ, PL/J and FVB/NJ, or MRL/MpJ and FVB/NJ,

respectively. There are uniquely regulated genes for PL/J, MRL/MpJ, or FVB/NJ, which number 28, 30, or 28, respectively. Of the 16 upregulated common genes among the 3 strains, it is reported that induction of nitric oxide synthase 2 (also known as inducible NOS) is related to the pathogenesis of hypoxic PH (33). Among the 14 commonly downregulated genes, Smad6 in the BMP signaling pathway is included. The mutation in the genes like BMPRII and ALK1 in this pathway are well known for the pathogenesis of PAH (5). Notably, we detected three genes that are upregulated (Igfbp2, Sparcl1, and Mctp1) and three genes that are downregulated (Npr3, Serpina3f, and Slc6a2), which are common between MRL/MpJ and FVB/NJ, but not in PL/J. This raises the question as to whether these six top regulated gene candidates, which are shared between MRL/MpJ and FVB/NJ, but not in PL/J, provide protective effects against PH, because MRL/MpJ and FVB/NJ showed a similar low-response phenotype.

Upregulation of miRNAs from the Dlk1-Dio3 Imprinting Region in PL/J after Hypoxia

Our analysis of miRNA-seq data from mice in both normoxia and various time points of hypoxia exposure (3 d, 1 wk, and 3 wk) identified 79 upregulated and 33 downregulated miRNAs (fold change [FC] ≥ 1.5 or ≤ -1.5 , $P < 0.05$). Using these differentially expressed miRNAs, we generated Venn diagrams shown in Figure 3A and Tables E6 and E7. Both upregulated and downregulated miRNAs

were common to either two or three hypoxia time points, as well as differentially expressed miRNAs unique to each time point. Our analysis also revealed that 43 of the 79 upregulated miRNAs mapped to the Dlk1-Dio3 imprinting region on chr12, whereas 2 of 33 downregulated miRNAs were mapped to the region (Tables E6 and E7). Heatmap analysis of miRNAs in the Dlk1-Dio3 imprinting region showed that 41 of 53 miRNAs were upregulated (FC ≥ 1.5) at 3-week hypoxia in PL/J, whereas only 5 of 53 and 14 of 53 miRNAs were upregulated in FVB/NJ and MRL/MpJ, respectively (Figure 3B and Figure E3).

To validate the miRNA expression, we focused on those miRNAs with an FC of 1.5 or greater and a P value less than 0.05 in PL/J, but not in MRL/MpJ and FVB/NJ, and then selected six miRNA candidates (miR-541-5p, miR-434-3p, miR-381-3p, miR-127-3p, miR-300-3p, and miR-411-5p) based on read counts. By quantitative PCR (qPCR), PL/J 3-week hypoxia showed FC increases ranging from 3.0 to 4.4 compared with PL/J normoxia, whereas MRL/MpJ showed FC increases ranging from 0.9 to 1.7 compared with MRL/MpJ normoxia (Figure 3C). Comparison of expression levels of the miRNAs between PL/J and MRL/MpJ after hypoxia showed five miRNAs (miR-541-5p, miR-434-3p, miR-381-3p, miR-127-3p, and miR-300-3p) in PL/J were significantly upregulated compared with MRL/MpJ, whereas the sixth miRNA (miR-411-5p) showed upregulation that did not reach statistical significance.

miRNAs Potentially Target T Cell Subsets and T Cell Subsets Are Downregulated in PL/J

To understand the role(s) of these six upregulated miRNAs, we searched for potential target mRNAs using TargetScan v7.1. Using 110 target mRNA candidates, we performed pathway analysis using DAVID (34, 35). The top hit was the pathway related with T cell subset surface markers, including Cd4, Cd8a, and Cd3d (Table E8). Using lung total RNA, qPCR analysis for these surface marker mRNA levels in MRL/MpJ and PL/J showed a significant decrease in PL/J 3-week hypoxics of, on average, 70% compared with normoxic controls, whereas MRL/MpJ hypoxics showed a smaller decrease, ranging from 20% to 40% compared with normoxic controls (Figure 4A). Comparison of

Figure 2. Venn diagram for overlap of upregulated or downregulated genes. Represented is the overlap of the top 50 upregulated (fold change [FC] ≥ 1.5 , left panel) and top 50 downregulated (FC ≤ -1.5 , right panel) genes observed in PL/J (green), MRL/MpJ (blue), and FVB/NJ (orange) at 3 weeks hypoxia exposure compared with normoxic controls.

Figure 3. MicroRNAs (miRNAs) from Dlk1-Dio3 imprinting region were upregulated after hypoxia exposure in PL/J. (A) Venn diagram representing overlap of upregulated ($FC \geq 1.5$, left panel) and downregulated ($FC \leq -1.5$, right panel) miRNAs observed in 3 days (pink), 1 week (blue), and 3 weeks (yellow) of hypoxia exposure compared with normoxic controls in the PL/J strain. (B) The heatmap for the 53 miRNAs derived from Dlk1-Dio3 regions observed in normoxia and 3 weeks of hypoxia exposure in the FVB/NJ, MRL/MpJ, and PL/J strains. Yellow represents downregulated miRNAs, whereas blue indicates upregulated miRNAs. (C) Relative expression levels of miRNAs (miR-541-5p, miR-434-3p, miR-381-3p, miR-127-3p, miR-300-3p, and miR-411-5p) determined by qRT-PCR at 3-week hypoxic exposure compared with normoxia. The gray and black bars show hypoxic MRL/MpJ and PL/J strains, respectively. Data are presented as mean $\pm$ SEM ($n = 3$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

expression levels between MRL/MpJ and PL/J showed three potential target mRNAs (Cd4, Cd8a, and Cd3d) in PL/J that were significantly downregulated after hypoxia compared with MRL/MpJ. There was a trend for Cd8b1 to be downregulated in PL/J as compared with MRL/MpJ, but this did not reach statistical significance. Our miRNA and mRNA qPCR results and *in silico* target prediction identifies three potential miRNA-target mRNA pairs: miR-381-3p and Cd3d; miR-541-5p and Cd4; and miR-381-3p and Cd8a.

FACS analysis was used to calculate the percentage of T cells ($CD4^+$, $CD8^+$,

and $CD4^+$ $CD8^+$ double-positive [DP] T cells) in total lung samples of PL/J and MRL/MpJ (Figure 4B). Allowing for lag in T cell production and development, we performed a 5-week hypoxia exposure. PL/J hypoxic animals showed significantly reduced percentages compared with normoxic controls: reduction of 61.8% for $CD4^+$ cells ($P < 0.01$), 44.6% for $CD8^+$ cells ($P < 0.001$), and 56.5% for DP T cells ($P < 0.01$). However, MRL/MpJ hypoxics showed similar percentages of $CD4^+$, $CD8^+$, and DP T cells as compared with normoxic controls. We also determined the percentage of T cells in the spleen, as this is one of the major sites of T cell activation.

The percentage of $CD4^+$, $CD8^+$, and DP T cells was significantly decreased compared with normoxic controls in PL/J, but MRL/MpJ showed only a slight reduction of $CD4^+$ cells and DP T cells, and a slight increase in $CD8^+$ cells, compared with normoxic controls (Figure 4C).

Increased DCs and C5a/C5AR1 Expression

We analyzed the percentage of DCs in the lung after a 5-week hypoxic exposure (Figure 5A). PL/J strain mice had a 5.2-fold increase in the percentage DCs after hypoxia ($P < 0.05$) as compared with

Figure 4. Downregulation of T cell-related mRNAs and T cell subsets. (A) Relative expression levels of mRNAs (Cd4, Cd8a, Cd8b1, and Cd3d) determined by qRT-PCR at 3 weeks of hypoxic exposure compared with normoxia. The gray and black bars show hypoxic MRL/MpJ and PL/J strains, respectively. Data presented as mean $\pm$ SEM ($n = 3$, $*P < 0.05$). To confirm the impact of hypoxia on T cell status, single-cell suspensions were prepared from (B) lung and (C) spleen of MRL/MpJ and PL/J mice (see METHODS). Absolute percentages of CD4⁺, CD8⁺, and CD4⁺CD8⁺ double-positive T cell subsets are shown from indicated tissues (B and C). Data are representative of three to five mice per group (mean $\pm$ SEM, $**P < 0.01$; $***P < 0.001$). The white, gray, and black bars show normoxia, MRL/MpJ hypoxia, and PL/J hypoxia, respectively.

normoxic PL/J, whereas MRL/MpJ showed a 3.4-fold increase ($P < 0.05$) compared with normoxic controls. The percentage of DCs in PL/J after hypoxic exposure is twofold higher compared with that of MRL/MpJ ($P < 0.05$).

To detect any hypoxia-dependent changes in expression of C5AR1 in the DCs of PL/J, we analyzed the level of C5AR1 in DCs by FACS analysis. In 5-week-hypoxia-exposed PL/J mice, the level of

C5AR1 was greatly increased (57.3-fold) compared with normoxic controls, whereas that of hypoxic MRL/MpJ mice demonstrated only a 1.7-fold increase compared with normoxic controls (Figure 5B). In alveolar lavage samples after 3 weeks of hypoxia, C5a was increased 185% compared with the normoxic controls in PL/J (Figure 5C). C5a was increased 57.2% after hypoxia compared with the normoxic controls in MRL/MpJ. The

baseline level of C5a was 37-fold higher in PL/J as compared with MRL/MpJ.

Because C5a was elevated, we determined whether expression of select proinflammatory cytokines (TNF- α , IL-1 β , and IL-6) in DCs was dependent on *in vitro* C5a treatment after isolation of DCs from lungs of hypoxia- or normoxia-treated animals (Figure 5D). In DCs with no C5a treatment, cells from PL/J 3-week hypoxic mice had higher concentrations of TNF- α ,

Figure 5. Hypoxia-exposed PL/J mice show increased incidence of pulmonary dendritic cells (DCs), upregulation of C5a and C5aR1, and their association with signature proinflammatory cytokines. (A) Single-cell suspensions, prepared from lungs harvested from MRL/MpJ and PL/J mice, were stained for DC-specific markers. The absolute percentage of CD11c⁺ CD11b⁺-positive DCs are shown. (B) FACS staining with antibodies to CD11b and CD11c was performed on DCs purified with CD11c microbeads. These DCs were gated for CD11c high positivity and stained for CD88/C5aR1 positivity. (C) Cells were collected via alveolar lavage from hypoxia-induced MRL/MpJ and PL/J mice and used to measure C5a by ELISA. (D) Pulmonary DCs purified from hypoxia-induced MRL/MpJ and PL/J mice were stimulated in the presence and absence of C5a (100 nM). Culture supernatants were measured for proinflammatory cytokines TNF- α , IL-1 β , and IL-6 by ELISA. Data are representative of 3–5 mice per group (mean $\pm$ SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Normoxia (white bar), MRL/MpJ hypoxia (gray bar), and PL/J hypoxia (black bar).

IL-1 β , and IL-6 compared with the normoxic controls ($P < 0.01$, $P < 0.001$, and $P < 0.01$, respectively). In DCs with C5a treatment, PL/J 3-week hypoxics showed greatly increased concentration of TNF- α , IL-1 β , and IL-6 compared with the normoxic controls ($P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively). However, we did not see such a pronounced induction of cytokines dependent on C5a treatment in hypoxic MRL/MpJ. This demonstrates that expression of proinflammatory cytokines is much greater in PL/J in the presence of C5a after hypoxia.

Rock2 Contributes to Vasoconstriction in PL/J Strain

Pathway analysis was performed using DAVID (34, 35) for those 528 mRNAs showing differential expression ($FC \geq 1.5$ or ≤ -1.5) compared with normoxic controls for each hypoxia exposure time point in PL/J, but not showing differential expression in either FVB/NJ or MRL/MpJ (Figure E4). In the 3-week hypoxia exposure, Rock2 ($FC = 1.7$) was included in the top four pathways: vascular smooth muscle contraction, extracellular matrix–receptor interaction, cGMP-PKG signaling pathway, and cell cycle pathways. Using qPCR, Rock2 mRNA expression was found to be increased in PL/J mice ($FC = 1.3$, $P < 0.05$) after 3 weeks of hypoxia compared with the normoxic controls. Rock2 expression remained largely unchanged ($FC = 1.0$, $P > 0.05$) in hypoxia compared with the normoxia controls in MRL/MpJ (Figure 6A).

To investigate potential miRNA regulation of Rock2 expression, we applied the following two criteria: 1) miRNAs that are downregulated after 3 weeks of hypoxia in the miRNA-seq data, as Rock2 mRNA is upregulated at this time point; and 2) miRNAs that potentially target Rock2 mRNA based on *in silico* prediction of target sites. Among five candidates that met these criteria, we selected miR-150-5p. miR-150-5p is downregulated at 3 weeks specifically in PL/J, and the average read counts of miR-150-5p was highest. In PL/J, qPCR showed that expression of miR-150-5p was downregulated twofold ($P < 0.01$) compared with normoxic animals, whereas that of MRL/MpJ was increased 1.3-fold compared with normoxic controls (Figure 6B). We observed an expected inverse correlation in expression of miR-150-5p and Rock2.

Figure 6. Rock2 affects RVSP via vasoconstriction. (A) Relative expression levels of Rock2 mRNAs and (B) miR-150-5p are measured by qRT-PCR. The gray and black bars show hypoxic MRL/MpJ and hypoxic PL/J, respectively. Data are presented as mean $\pm$ SEM ($n = 3$; * $P < 0.05$, ** $P < 0.01$). (C) RVSP was measured in PL/J mice exposed to 4 weeks of hypoxia using right heart catheterization before and after treating with either Fasudil or Y-27632 by intraperitoneal injection. Saline-treated mice were used as control. Data presented as mean $\pm$ SEM; $n = 5$ (saline, hypox), $n = 15$ (Fasudil, norm), $n = 14$ (Fasudil, hypox), $n = 12$ (Y-27632, norm), and $n = 14$ (Y-27632, hypox) (** $P < 0.001$). Rock2 = Rho-associated protein kinase 2.

To determine the contribution of Rock-dependent vasoconstriction to the increase in RVSP, we measured RVSP in hypoxia-exposed PL/J by right heart catheterization both before and after treatment with either of two Rock inhibitors, Fasudil or Y-27632, during catheterization (Figure 6C). In the hypoxic animals, measured RVSP after treatment with Fasudil or Y-27632 for 5 minutes was significantly reduced compared with predrug treatment (Fasudil, 31.9% reduction, $P < 0.001$; Y27632, 31.6% reduction, $P < 0.001$). In normoxic PL/J, RVSP was reduced after treating with either inhibitor (Fasudil, 14.1% reduction, $P < 0.001$; Y27632, 21.1%, $P < 0.001$). In the saline-treated animals, little change in RVSP was observed between before and after treatment groups (1.8% reduction, $P > 0.05$). These data suggest that vasoconstriction accounts for approximately 32% of the increase in RVSP measured in PL/J animals after exposure to chronic hypoxia.

Discussion

The present study is the first large-scale investigation to identify hypoxia-induced PH-susceptible and -resistant inbred mouse strains. Using these strains, the long-term goal is to identify novel genes/pathways that contribute to the pathogenesis of human PAH. Our studies found that the PL/J strain

exhibited by far the largest increase in RVSP in response to hypoxia exposure with an average RVSP of 86.58 mm Hg as compared with 31 other inbred strains. In addition, PL/J developed significant RVH, modest lung remodeling, and an inflammatory response phenotype including decreased numbers of CD4⁺, CD8⁺, and DP T cells and an increased number of DCs. A comparison of gene expression in lung mRNA after hypoxic exposure by mRNAseq analysis between PL/J and two resistant low-responder strains (FVB/NJ and MRL/MpJ) identifies both upregulated and downregulated genes that are shared between all three strains as well as genes unique to each of the individual strains. We hypothesized that, because MRL/MpJ and FVB/NJ strains both exhibit resistance to hypoxia-induced PH, these two strains may show similar changes in gene expression as compared with PL/J. Of the 50 most upregulated and 50 most downregulated genes, we identified 3 for upregulation (Igfbp2, Sparcl1, and Mctp1) and 3 for downregulation (Npr3, Serpina3f, and Slc6a2) in common between MRL/MpJ and FVB/NJ that are not identified in PL/J. Although it is possible that any or all of these six genes commonly regulated by hypoxia in these two strains confer protection against PH, it seems more likely that changes in gene expression unique to PL/J confer sensitivity to PH, as this was the only strain among the 32 studied demonstrating extreme sensitivity to

hypoxia. However, genes that confer protection from PH could be an alternative avenue of investigation in the future.

This is the first report of increased expression of miRNAs clustering in the Dlk1-Dio3 imprinted region on chr12 in a hypoxia-induced PH mouse model. Changes in expression of the miRNAs clustering in this region have been observed in other diseases in both mouse models and human patients; this imprinted region is conserved between mouse chr12 and human chr14 (36, 37). In one report, some miRNAs from the Dlk1-Dio3 region were upregulated compared with control mice in splenocytes of the murine lupus model (38). In another, the miRNA cluster was downregulated in islets from type 2 diabetes mellitus organ donors (39). Some miRNAs from the Dlk1-Dio3 region are upregulated in atherosclerosis (40). miRNA expression changes in this region can be associated with DNA methylation, and so we speculate that miRNA expression changes observed in our study could potentially involve DNA methylation.

In silico analysis of mRNAs encoding cell surface markers for T cells have target sites of these upregulated miRNAs in the Dlk1-Dio3 imprinted region. These mRNAs were downregulated in PL/J after hypoxic exposure. In addition, the number of T cells was decreased after hypoxic exposure in PL/J. One of the target genes exhibiting decreased expression, Cd3d, is essential for the

development of T cells (41), suggesting that miR-381-3p, which was predicted to target Cd3d mRNA, may repress mRNAs encoding T cell subsets, preventing their development. T cell deficiency can confer a PAH phenotype (19), and depletion of CD4⁺ cells can increase PAH susceptibility (21). Because our data show a decrease in the number of CD4⁺ T cells in hypoxic PL/J in lung and spleen, it is possible that this may be contributing to the development of the phenotype. Taken together, our data suggest that expression of miRNAs from the Dlk1-Dio3 imprinted region may regulate T cell abnormalities in hypoxic PL/J, leading to a PAH-like phenotype.

A previous study demonstrated accumulation of immature DCs in remodeled pulmonary arteries of human and experimental PH (22). Interestingly, we also found an increased number of DCs in the lungs of hypoxia-exposed PL/J compared with normoxic controls. Previous studies have reported that overexpression of C5AR1 promotes morphological changes of gastric cancer cells by activating RhoA (42), and that C5a-activated polymorphonuclear leukocytes activate RhoA in bovine coronary venular endothelial cells (43). In addition, RhoA activates Rock (44). As we observed the expression of Rock2 in lung tissue was significantly increased in hypoxia-exposed PL/J, we suggest that the increased expression of C5a and C5AR1 in PL/J hypoxia-exposed lung leads to increased Rock2 expression via RhoA. The RhoA/Rho kinase pathway is known to play a role in vasoconstriction in PH (45, 46).

Inhibition of Rock2 by Fasudil or Y-27632 during catheterization in hypoxia-exposed PL/J reduced RVSP by approximately 32%. The significant increase in RVSP in PL/J versus MRL/MpJ after hypoxia being much greater than the pulmonary artery remodeling, when combined with the Rock2 mRNA expression and *in vitro* inhibitor data, suggests that PL/J may have a stronger vasoconstrictor response than MRL/MpJ. Rock-dependent vasoconstriction is involved in elevated RVSP in other PAH studies (47). Several studies have shown that Rock2 inhibition can ameliorate PAH symptoms, and that Rock inhibitors might be therapeutic targets for PAH (45, 46). Furthermore, our study suggests that

the PL/J strain has stronger vasoconstrictor response because RVSP increased more in PL/J than MRL/MpJ than pulmonary arterial remodeling. The data of Rock2 expression and Rock inhibition support this idea. Based on our results and those of previous studies (42–47), we suggest that elevation of C5a and its receptor, C5AR1, may lead to Rock2 mRNA increases via the RhoA pathway, resulting in RVSP increases in PL/J. Because C5a and C5AR1 are components of the complement pathway, it is possible that complement activation may contribute to the development of hypoxia-induced PAH, although additional studies are needed to determine causality. Deficiency of C3, another factor in the complement pathway, attenuated hypoxia-induced PH in mice (23). If C5a and/or C5AR1 can regulate RVSP indirectly, these could

potentially be novel therapeutic targets for the treatment of PAH.

To identify additional potential genomic factors that could be associated with elevated RVSP in hypoxia-exposed PL/J animals, our analysis of miRNA expression pointed to miR-150-5p. miR-150-5p was decreased in PL/J after hypoxia compared with normoxic controls, and unchanged in MRL/MpJ. Interestingly, *in silico* analysis identified target sites for miR-150-5p on Rock2 mRNA, which is elevated in PL/J after hypoxic exposure. We hypothesize that miR-150-5p downregulation results in increased Rock2 expression, promoting vasoconstriction and contributing to elevated RVSP in PL/J. Previous studies have also shown reduced expression of miR-150-5p in plasma from patients with PAH and in lungs of monocrotaline-treated rats (17), as well as in hypoxia-treated pulmonary artery

Figure 7. Possible mechanisms contributing to elevated RVSP in PL/J pulmonary arterial hypertension model. We identified two possible pathways contributing to the elevation of RVSP in PL/J mice based on our observations in this study. In the first pathway, hypoxia drives miRNA upregulation from the Dlk1-Dio3 imprinted region, causing downregulation of mRNAs related to T cell surface marker and development, and resulting in downregulation of the T cell numbers. In the second pathway, hypoxia drives upregulation of dendritic cells, initiating upregulation of C5a and its receptor, C5AR1, resulting in downregulation of miR-150-5p and upregulation of Rock2. The red arrows show the up- or downregulation of the expression of miRNAs, mRNAs, or cells validated in this study. The solid black arrow indicates the phenomenon that we validated, whereas the dashed black arrows indicate the speculation to be confirmed in future studies.

smooth muscle cells (48). Hence, it is possible that there is a role for miR-150-5p in PAH susceptibility/development.

In summary, we identified two potential mechanisms/pathways contributing to increased RVSP and the development of PH in the PL/J strain (Figure 7). We have shown increased expression of miRNAs in the Dlk1-Dio3 imprinted region, which may regulate expression of T cell development, resulting in a reduction of T cells in PL/J. We have also identified changes in C5a/C5AR1 as well as miR-150-5p, both of which may lead to an elevation in Rock2 expression, causing vasoconstriction of the lung

pulmonary arteries, resulting in elevated RVSP. Additional studies are necessary to confirm the role of increased miRNA expression in the reduction of T cell populations, as well as the role of increased C5a and C5AR1 in DCs and any association with elevated Rock2 expression and elevated RVSP, resulting in hypoxia-induced PH. Limitations of our study include lack of data demonstrating changes of inflammatory cell mediators and the inflammatory components in a time-dependent manner, as well as cell counts in the cell populations reported. However, our findings in PL/J support previous studies of both T cell depletion and miR-150-5p as

factors contributing to PAH development. The PL/J strain may provide an important animal model for seeking mechanisms of elevated RVSP hypoxia-induced PAH for both studies of disease pathogenesis and identifying potential novel treatment targets that may be translatable to the human disease. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank Chelsea DeAne Tolentino, Hua He, and Stuart Tinch from Cincinnati Children's Hospital Medical Center for excellent technical assistance and statistical advice.

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