Lung miRNA profiles show a time-of-day response in house dust mite-induced allergic asthma in vivo

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Lung miRNA profiles show a time-of-day response in house dust mite-induced allergic asthma *in vivo*

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To the Editor

Asthma is a chronic inflammatory lung disease that shows a time-of-day effect (airway inflammation and lung function) on the severity of the disease [1]. Recent studies support the functional role of miRNAs in the molecular pathophysiology of asthma phenotypes/endotypes [2,3] through post-transcriptional gene regulation of key signaling pathways and cellular processes in human airways and immune cells of asthmatics and mouse model of asthma[4,5,6]. However, whether rhythmic changes in the expression of miRNAs are responsible for the time-of-day effects observed during asthma and its exacerbations is not known.

In this study, we performed miRNA and mRNA expression profiling using NanoString to determine the time-of-day effects using house dust mite (HDM)-induced allergic asthma model. Differential expression (DE) analysis revealed a strong time-of-day difference in the miRNA expression at ZT0 and ZT12 in HDM vs. PBS (control), as shown by hierarchical cluster analysis (**Figure 1A-D**). We found 6 miRNAs (downregulated: miR-125b-5p, miR-125a-5p, miR-150, miR-23a, miR-23b and miR-15b) and 3 miRNAs (upregulated: miR-652, miR-200b and miR-200c) significantly in HDM vs. PBS at ZT0 (**Figure 1A-B**), and 21 miRNAs downregulated and 17 miRNAs upregulated significantly in HDM vs. PBS at ZT12 (**Figure 1C-D**).

When we compared the DE miRNAs in HDM vs. PBS group at ZT0 and ZT12, we found the same 6 miRNAs (downregulated) and 3 miRNAs (upregulated) at ZT0 pairwise comparisons were present (**Figure S1A-B**). Additionally, unsupervised clustering and PCA of normalized counts from HDM vs. PBS at ZT0 and ZT12 showed clusters separated based on the treatment groups (**Figure S1C-D**). Hierarchical cluster analysis of HDM vs. PBS groups at ZT0 and ZT12 together revealed close clustering of all DE miRNAs in PBS and HDM groups at ZT12 except for few HDM ZT0 samples that were clustered with either the PBS ZT0 or HDM ZT12 (**Figure S1E**).

Multiple comparison analyses revealed several miRNAs that were either significantly downregulated or upregulated in HDM vs. PBS at ZT0 and ZT12 (**Figure 2A** and **Figure S2A-B**). We found about 11 miRNAs downregulated and 8 miRNAs upregulated significantly in HDM vs. PBS at ZT12, and 3 miRNAs were upregulated in HDM vs. PBS at ZT0 (**Figure S2A-B**). However, several miRNAs were either downregulated (2 miRNAs: miR-720 and miR-1944) or upregulated (9 miRNAs: miR-151-5p, miR-199a-3p, miR-30c, let-7d, miR-15a, miR-181a, miR-25, let-7e, and miR-126-3p) in PBS ZT0 vs. ZT12 comparison (**Figure S2A-B**). The pairwise comparison between HDM vs. PBS at ZT0 and ZT12 revealed 9 miRNAs and 38 miRNAs DE at ZT0 and ZT12 respectively (**Figure S3A-C**). Additional pairwise comparison between PBS at ZT0 vs. ZT12 and HDM ZT0 vs. ZT12 revealed several miRNAs DE were among those identified in HDM vs. PBS at ZT12 (**Figure S1F**).

NanoString Myeloid Innate Immunity panel was utilized to validate the miRNA-mRNA targets predicted by Ingenuity Pathway Analysis (IPA). The predicted mRNA target genes for DE miRNAs were validated based on normalized counts and qPCR analysis (**Figure 2B** and**Figure S4A**). The predicted mRNA target genes include*il13, tnfrsf1b, arg1, fgf2, fut4, ccr2, vamp2, adamts3, cxcl10, cxcr5, yap1, ptgs2, ikbke, hla-a, prg2, lat2, tlr2, pdcd1lg2,* and*usp18* . These miRNA-mRNA predictions were based on IPA and miRPath analyses and their gene annotation to specific signaling pathways including the cell type associated with allergic asthma are summarized (**Table S1, Figure 2C, and Figure S4A-C**). We speculate that the time-of-day difference in DE miRNAs in allergic asthma (ZT0 vs. ZT12) may be due to disruption of circadian rhythms in the lung which were among the KEGG pathway predicted showing >1 miRNAs interacting with 15 circadian genes (**Figure S4C**).

Overall, we found a significant time-of-day difference in the DE of miRNAs in HDM vs. PBS exposed mouse lungs at ZT0 and ZT12. DE miRNA and IPA-predicted mRNA target genes belong to cytokine/chemokine signaling, growth factor signaling, Th2 activation, etc. that was further validated by NanoString and qPCR analyses. miRPath analysis support our findings from DE miRNA-mRNA, which were linked to KEGG pathways directly associated with asthma and inflammation. This is the first report that shows a time-of-day difference in miRNA expression in the lung following HDM exposure that may involve the interaction of circadian clock genes which need to be further investigated.

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FIGURE LEGENDS:

Figure 1. miRNA expression signatures in PBS (control) and house dust mite (HDM) exposed mouse lung. (A) Heatmap shows unsupervised hierarchical clustering of nine differentially expressed (DE) miRNAs in HDM vs. PBS exposed mouse lung at ZT0 analyzed by Morpheus tool. **(B)** Pairwise comparison

analysis performed by nSolver 4.0 software showing DE miRNAs in HDM vs. PBS at ZT0.**(C)** Heatmap shows unsupervised hierarchical clustering of thirty-eight DE miRNAs in HDM vs. PBS exposed mouse lung at ZT12 analyzed by Morpheus tool. **(D)** Pairwise comparison analysis performed by nSolver 4.0 software showing DE miRNAs in HDM vs. PBS at ZT12. Data are shown as fold change/ratio, P -value ($P< 0.05$) and FDR adjusted P -value < 1 (n=6/group/time point).

Figure 2. Representative data from differentially expressed miRNAs and selected mRNA targets were analyzed by NanoString nCounter analysis. **(A)** DE 9 miRNAs were significantly downregulated at ZT0 and ZT12 pairwise comparison. We observed a time-of-day effect in the expression of miRNAs in HDM vs. PBS exposed mouse lung. **(B)**Selected miRNA-mRNA targets predicted by Ingenuity Pathway Analysis were further validated using NanoString mouse Myeloid Innate Immunity Panel and qPCR analysis (*il13* mRNA). Normalized counts data are presented as analyzed by nSolver 4.0 software. **(C)**Bioinformatic analysis of DE miRNAs downregulated or upregulated in HDM exposed group at ZT12 using DIANA-miRPath v3.0. KEGG pathways marked in red color were pathways identified by miRPath analysis of DE miRNAs in HDM vs. PBS at ZT12. Data are shown as mean ± SEM (n=6/group; * $P< 0.05$, ** $P < 0.01$, *** $P < 0.001$, significant compare to respective PBS control;$^{\#}$ $P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}$ $P< 0.001$, significant compared to PBS or HDM from ZT0; 2way ANOVA with Tukey's multiple comparison test).

Abbreviations:

DE Differential Expression

FDR False Discovery Rate

FoxO Forkhead box O

HDM House Dust Mite

IPA Ingenuity Pathway Analysis

KEGG Kyoto Encyclopedia of Genes and Genomes

MAPK Mitogen-Activated Protein Kinase

miRNA MicroRNA

PBS Phosphate Buffered Saline

PCA Principal Component Analysis

PI3K-AKT Phosphoinositide 3-Kinase-Protein Kinase B

ZT Zeitgeber Time

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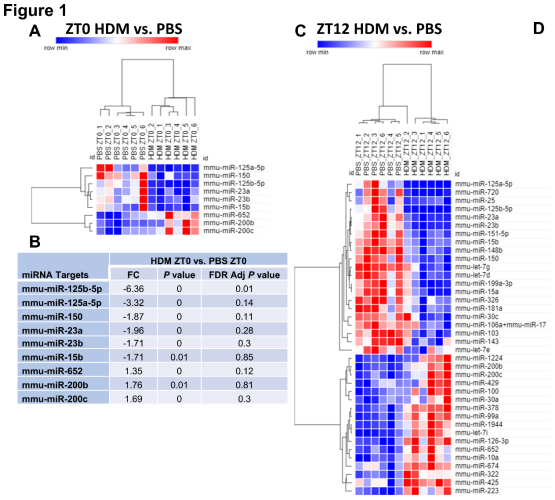
Figure 1



B

miRNA Targets	HDM ZT0 vs. PBS ZT0		
	FC	*P* value	FDR Adj *P* value
mmu-miR-125b-5p	-6.36	0	0.01
mmu-miR-125a-5p	-3.32	0	0.14
mmu-miR-150	-1.87	0	0.11
mmu-miR-23a	-1.96	0	0.28
mmu-miR-23b	-1.71	0	0.3
mmu-miR-15b	-1.71	0.01	0.85
mmu-miR-652	1.35	0	0.12
mmu-miR-200b	1.76	0.01	0.81
mmu-miR-200c	1.69	0	0.3

D

miRNA Targets	HDM ZT12 vs. PBS ZT12		
	FC	*P* value	FDR Adj *P* value
mmu-miR-125b-5p	-17.18	0	0
mmu-miR-125a-5p	-6.63	0	0
mmu-miR-720	-5.51	0	0.01
mmu-miR-23a	-4.23	0	0
mmu-miR-23b	-3.1	0	0
mmu-miR-150	-2.96	0	0.04
mmu-miR-15b	-2.71	0	0.07
mmu-miR-151-5p	-2.69	0	0.02
mmu-let-7g	-2.57	0.01	0.22
mmu-miR-199a-3p	-2.35	0	0.07
mmu-miR-30c	-2.28	0.03	0.84
mmu-let-7d	-2.02	0	0.08
mmu-miR-15a	-1.97	0	0.07
mmu-miR-148b	-1.88	0	0.01
mmu-miR-326	-1.62	0.01	0.2
mmu-miR-181a	-1.61	0	0.15
mmu-miR-25	-1.54	0.02	0.42
mmu-miR-103	-1.51	0	0.05
mmu-miR-143	-1.51	0.01	0.2
mmu-let-7e	-1.26	0.02	0.6
mmu-miR-106a+mmu-miR-17	-1.33	0.02	0.63
mmu-miR-1224	3.05	0	0.11
mmu-miR-378	2.25	0	0
mmu-miR-1944	2.13	0	0
mmu-miR-99a	2.02	0	0
mmu-miR-652	1.96	0	0.02
mmu-miR-200b	1.92	0	0.01
mmu-let-7i	1.83	0	0
mmu-miR-100	1.83	0.01	0.16
mmu-miR-200c	1.81	0	0.1
mmu-miR-322	1.76	0	0.02
mmu-miR-30a	1.73	0.01	0.32
mmu-miR-126-3p	1.55	0	0
mmu-miR-674	1.51	0.01	0.22
mmu-miR-10a	1.51	0	0.03
mmu-miR-223	1.41	0	0.04
mmu-miR-429	1.38	0.03	0.86
mmu-miR-425	1.33	0	0.15

Figure 2