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Isoprene-Derived Secondary Organic Aerosol Induces the Expression of MicroRNAs Associated with Inflammatory/Oxidative Stress Response in Lung Cells

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Abstract

Exposure to fine particulate matter (PM_{2.5}), of which secondary organic aerosol (SOA) is a major constituent, is linked to adverse health outcomes, including cardiovascular disease, lung cancer, and preterm birth. Atmospheric oxidation of isoprene, the most abundant nonmethane hydrocarbon emitted into Earth's atmosphere primarily from vegetation, contributes to SOA formation. Isoprene-derived SOA has previously been found to alter inflammatory/oxidative stress genes. MicroRNAs (miRNAs) are epigenetic regulators that serve as post-transcriptional modifiers and key mediators of gene expression. To assess whether isoprene-derived SOA alters miRNA expression, BEAS-2B lung cells were exposed to laboratory-generated isoprene-derived SOA constituents derived from the acid-driven multiphase chemistry of authentic methacrylic acid epoxide (MAE) or isomeric isoprene epoxydiols (IEPOX) with acidic sulfate aerosol particles. These IEPOX- and MAE-derived SOA constituents have been shown to be measured in large quantities within PM_{2.5} collected from isoprene-rich areas affected by acidic sulfate aerosol particles derived from human activities. A total of 29 miRNAs were identified as differentially expressed when exposed to IEPOX-derived SOA and 2 when exposed to MAE-derived SOA, a

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ASSOCIATED CONTENT

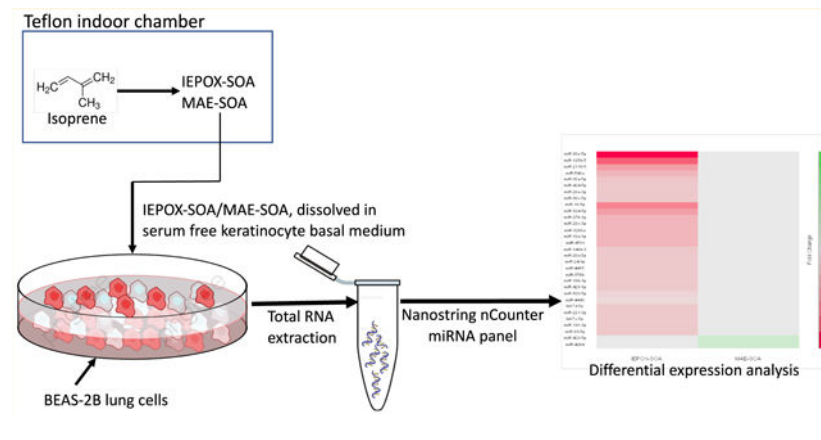
Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.chemrestox.9b00322>.

Supplemental figures depicting molecular networks (PDF)

number of which are inflammatory/oxidative stress associated. These results suggest that miRNAs may modulate the inflammatory/oxidative stress response to SOA exposure, thereby advancing the understanding of airway cell epigenetic response to SOA.

Graphical Abstract



1. INTRODUCTION

Exposure to atmospheric fine particulate matter ($\text{PM}_{2.5}$, aerosol with aerodynamic diameter $<2.5 \mu\text{m}$) is associated with significant adverse health outcomes such as cardiovascular disease,^{1–3} respiratory system damage and lung cancer^{4–11} as well as preterm birth.^{12–15} $\text{PM}_{2.5}$ is a heterogeneous mixture of organic and inorganic solid and/or liquid particles suspended in air.^{15,16} A dominant component of $\text{PM}_{2.5}$ is secondary organic aerosol (SOA), the majority of which is formed through gas-phase photooxidation (i.e., hydroxyl radical (OH)-initiated oxidation) of isoprene (2-methyl-1,3-butadiene).^{16–19} Isoprene, released primarily from broad-leaf trees, is the most abundant nonmethane hydrocarbon emitted into Earth's atmosphere ($\sim 600 \text{ Tg year}^{-1}$).²⁰ The interaction of gas-phase isoprene-derived oxidation products with anthropogenic pollutants, in particular acidic sulfate aerosol derived from combustion sources, produces isoprene-derived SOA.¹⁷ Thus, the composition of isoprene-derived SOA is strongly influenced by controllable anthropogenic emissions.²¹ Intermediates in isoprene-derived SOA formation, including isomeric isoprene epoxydiols (IEPOX), isoprene hydroxyhydroperoxide (ISOPOOH), and methacrylic acid epoxide (MAE), are critical gas-phase SOA precursors.²¹

$\text{PM}_{2.5}$ -induced oxidative stress plays a key role in respiratory system damage caused by air pollution.^{22,23} Oxidative stress, an oxidant/antioxidant imbalance in favor of oxidants, is a key driver of injury and inflammatory response in respiratory diseases, including asthma and chronic obstructive pulmonary disease.^{24,25} Given its size, $\text{PM}_{2.5}$ can completely penetrate the lungs, resulting in direct exposure of lung cells.²² Our previous work has shown that isoprene-derived SOA induced the expression of oxidative stress and inflammation genes in human lung cells.^{16,21,26,27} Of the SOA components tested in these previous studies, MAE-derived SOA was identified as being a more potent inducer of inflammatory and oxidative stress gene expression response than IEPOX-derived SOA or ISOPOOH-derived SOA.²¹

While the gene activation studies are important in providing insight into the cellular response to isoprene-derived SOA, other aspects of the regulatory pathway remain to be elucidated' namely post-transcriptional regulation of mRNA.

MicroRNAs (miRNAs) are noncoding single-stranded RNA molecules approximately 22 nucleotides in length.²⁸ miRNAs serve as post-transcriptional modifiers and key mediators of gene expression. Thus, they act as epigenetic regulators of genomic response to environmental exposures. In general, miRNAs interact with complementary regions of target mRNAs to induce mRNA degradation and repress translation.²⁹ In some cases, however, miRNAs have been shown to activate translation.²⁹

We hypothesized that exposure to isoprene-derived SOA would alter the expression of miRNAs that modulate genes previously found to be responsive to isoprene-derived SOA exposure. To test this hypothesis, we exposed BEAS-2B cells to both MAE- and IEPOX-derived SOA and evaluated sub-sequent miRNA expression relative to a control exposure. These results provide molecular level evidence linking isoprene-derived SOA exposure and observed system level respiratory outcomes.

2. EXPERIMENTAL METHODS

Synthesis of SOA Precursors.

Experimental procedures for the synthesis and characterization of SOA precursors *trans*- β -IEPOX and MAE and extraction of SOA constituents have been published previously.^{16,30,31} Identity and purity (>99%) of the precursors was confirmed by ¹H and ¹³C nuclear magnetic resonance (NMR) as well as by gas chromatography/electron ionization mass spectrometry (GC/EI-MS) analysis with prior trimethylsilylation (TMS) or ultraperformance liquid chromatography coupled to electrospray ionization high-resolution quadrupole time-of flight mass spectrometry (UPLC/ESI-HR-QTOFMS).^{30,31}

Generation and Chemical Characterization of IEPOX- and MAE-Derived SOA.

To generate SOA, authentic *trans*- β -IEPOX or MAE was injected into a 10-m³ flexible Teflon indoor chamber in the presence of acidic sulfate aerosol. This was conducted under dark conditions; therefore, radicals and peroxides were not expected to be present. Detailed operating procedures for this chamber facility for the purpose of *in vitro* exposures have been described previously.^{16,30,32} Filter extraction procedures have been described elsewhere.³² In brief, efficiency of removal of IEPOX- and MAE-derived SOA from filters was estimated to be over 90%. Filter samples were chemically characterized by GC/EI-MS and UPLC/ESI-HR-QTOFMS as described previously.¹⁶ Detailed sample preparation, column conditions, and operating parameters for GC/EI-MS and UPLC/ESI-HR-QTOFMS have been published elsewhere.^{33,34}

Cell Culture.

BEAS-2B cells were cultured in keratinocyte growth medium (KGM) (KGM BulletKit, Lonza), which contains serum-free KBM, bovine pituitary extract, human epidermal growth factor, insulin, hydrocortisone, and GA-1000 (gentamycin, amphotericin B) and grown at

37 °C and 5% CO₂ in a humidified incubator. A serum-free medium was chosen to maximize comparability to other studies evaluating *in vitro* exposure to isoprene-derived SOA, to follow ACCT recommendations, and because studies have noted that serum-exposed BEAS-2B cells are not an ideal model of the epithelial phenotype.^{16,21,26,35,36}

Extraction of SOA Constituents for Cell Exposure.

Sonication in high-purity methanol was used to extract the Teflon filter membranes. Several filter extracts were combined to achieve the desired dose level, then dried under a gentle stream of nitrogen and redissolved in growth factor-deprived serum-free keratinocyte basal medium (KBM). The acidified sulfate aerosol-only control filters were processed in the same way. SOA constituents are known to be highly methanol and water-soluble, and thus, the SOA constituents can be assumed to have been fully dissolved in the cell medium.³⁷

Cell Exposure.

For the exposure, cells were seeded in 24-well plates at a density of 2.5×10^4 cells/well in 250 μL of KGM 2 days before exposure. At 60–70% confluence, cells were washed twice with the phosphate-buffered saline (PBS) and then exposed for 24 h to KBM medium containing 0.01, 0.1, and 1 mg/mL SOA. This procedure was repeated for extracts of the acidic sulfate aerosol control filters. Experiments were conducted in triplicate per treatment group.

Assessment of Cytotoxicity.

To ensure that toxicity of exposure levels would not affect gene expression, toxicity at each exposure level was assayed using a lactate dehydrogenase (LDH) cytotoxicity detection kit (Takara Bio, Mountain View, California) according to the manufacturer's protocol. After being exposed for 24 h, the supernatants were collected to assess LDH levels. Cells exposed to filter extracts from acidified sulfate aerosol-only experiments and cells maintained in KBM alone were treated as control groups.

miRNA Extraction.

Cells were lysed with 350 μL of Trizol Reagent (Life Technologies, Carlsbad, California) after 24 hours for total RNA isolation.¹⁶ Spin column-based Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, California) was used to purify the RNA samples. RNA quantity was determined with Nanodrop (Thermo Scientific, Wilmington, Delaware) and quality with Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California). Total RNA was hybridized to the nCounter Human v3 miRNA panel comprising 798 human miRNA targets and 5 housekeeping genes (NanoString Technologies, Inc., Seattle, Washington). The NanoString hybridization was performed by the Translational Genomics Laboratory at the UNC at Chapel Hill Lineberger Comprehensive Cancer Center.

Statistical Analysis.

Raw expression data were extracted using the nSolver Analysis Software 4.0 (NanoString Technologies, Inc., Seattle, Washington) and normalized both to positive controls and housekeeping genes as recommended by the manufacturer.³⁸ miRNA expression changes in

cells exposed to SOA constituents were compared to changes in cells exposed to the extracts from acidic sulfate aerosol controls to assess the effects induced solely by the extracted SOA constituents using log fold changes calculated via an ANOVA model. Log fold changes are defined as the value of the geometric mean of the normalized miRNA expression in the cells exposed to 0.1 mg/mL IEPOX- or MAE-derived SOA, divided by the geometric mean of the normalized miRNA expression in the cells exposed to 0.1 mg/mL acidified sulfate aerosol. This was conducted in Partek Genomics Suite software (Partek Inc., St. Louis, MO, United States) and significant differential expression was defined as a log fold change with an associated false discovery rate adjusted $q < 0.05$. Ingenuity Pathway Analysis (IPA) software miRNA Target Filter tool (Ingenuity Systems, Inc., Redwood City, CA, United States) was used to identify two comprehensive lists of computationally predicted gene targets of the miRNAs differentially expressed in response to IEPOX- and MAE-derived SOA, respectively. Expression pairs were defined as miRNA-mRNA pairs in which the miRNA's computationally predicted gene targets were also found to be differentially expressed in gene expression studies of isoprene-SOA exposure.^{16,21} To validate the predicted gene target, we queried the European Bioinformatics Institute's Expression Atlas' "RNA-seq analysis of 2 cell line models of lung disease (A549 and BEAS-2B) and primary bronchial epithelial cells in Project 3 of Open Targets 020", which contained mRNA baseline data.³⁹ Additionally, network analysis to identify enriched biological pathways associated with the miRNAs differentially expressed was conducted using IPA software (Ingenuity Systems, Inc., Redwood City, CA, United States). Cellular networks representing perturbed pathways were identified through enrichment analysis performed using the Fisher's Exact test as detailed previously⁴⁰. Over-represented diseases and/or biological functions were defined as those associated with more gene product targets than expected by chance using a p -value < 0.01 .

3. RESULTS

Exposure Characterization.

Detailed exposure conditions have been described elsewhere, including description of aerosol chemical composition analysis.¹⁶ SOA mass yields from the reactive uptake of *trans*- β -IEPOX onto acidified sulfate aerosol are substantially larger than those from reactive uptake of MAE under the same experimental conditions ($< 10\%$ RH) and time scale (2 hour reaction time), which is consistent with ambient measures from the southeastern United States.⁴¹ Additionally, the SOA constituents found in the filter extracts of IEPOX- and MAE-derived SOA were also observed in ambient PM_{2.5} samples collected from isoprene-rich regions like the southeastern United States, which further supports the validity of the chamber experiments as representative of ambient SOA composition.^{16,27,41} The amount of SOA material the cells were exposed to was also relevant to a human ambient acute exposure. Ambient isoprene-derived SOA mass concentrations can range from 500 ng/m³ to 10–16 $\mu\text{g}/\text{m}^3$.^{42,43} If a worst-case exposure scenario of 16 $\mu\text{g}/\text{m}^3$ for 8 hours is assumed, with an inhalation rate of 6 L/min (i.e., 12 breaths per minute and the tidal volume ~ 0.5 L), then ambient exposure for 8 hours is 16 $\mu\text{g}/\text{m}^3 \times 6 \text{ L}/\text{min} \times 60 \text{ min}/\text{h} \times 8 \text{ h} \times 0.001 \text{ m}^3/\text{L} = 46.08 \mu\text{g} = 0.046 \text{ mg}$.

Cytotoxicity Measurements.

Cells exposed to a level of 0.1 mg/mL were selected for miRNA expression analysis as this dose was determined to be noncytotoxic. Significant cell death was observed in acidified sulfate aerosol-only controls at a concentration of 1 mg/mL (cell death, ~27%; $p = 0.02$), while acidified sulfate aerosol-only controls at concentrations of 0.1 mg/mL were not cytotoxic (cell death, ~10%; $p > 0.05$).

Altered Expression of miRNAs Induced by Exposure to Isoprene-Derived SOA.

A total of 29 miRNAs were identified that displayed differential expression when exposed to IEPOX-derived SOA (Table 1). Two miRNAs were identified that were differentially expressed when exposed to MAE-derived SOA (Table 1). IEPOX-derived SOA exhibited a more robust response in absolute magnitude of expression log fold changes and number of miRNAs differentially expressed (Figure 1). All miRNAs differentially expressed in response to IEPOX-derived SOA were down-regulated (negative log fold change), while the two miRNAs differentially expressed in response to MAE-derived SOA were up-regulated (positive log fold change). miRNAs differentially expressed in response to IEPOX-derived SOA exhibited greater absolute log fold changes than those in response to MAE-derived SOA. The largest log fold change in response to MAE-derived SOA was +2.99, versus -12.67 in response to IEPOX-derived SOA.

Network Analysis and miRNA-mRNA Expression Pairing.

There was substantial overlap in the computationally predicted gene targets of miRNAs that were differentially expressed in the current study and genes that we have previously found to be differentially expressed in response to isoprene-derived SOA exposure.^{16,21} For IEPOX-derived SOA, 52 gene target-miRNA expression pairings were identified (Table S1). Forty-five of these pairs showed an inverse expression pattern, suggestive of miRNA repression of mRNA levels. For MAE-derived SOA, 7 gene target-miRNA expression pairings were identified, 6 of which involved miR-423-5p (Table S2). For these expression pairs responsive to MAE-derived SOA exposure, both miRNA and mRNA demonstrated increased expression in response to exposure, counter to the expected pattern. Of the 59 total miRNA-mRNA expression pairs identified, 55 of the mRNA genes are known to be expressed in BEAS-2B cells according to baseline RNA-sequencing, thus providing confidence in the computationally predicted pairings (Tables S1 and S2).

Network analysis revealed that of the 59 total miRNA-mRNA pairings identified as responsive to IEPOX-derived SOA, 33 of the pairings had NRF2-mediated oxidative stress response as one of their associated pathways. Given that there were only 2 miRNAs responsive to MAE-derived SOA, network analysis was not performed. However, the 29 miRNAs differentially expressed when exposed to IEPOX-derived SOA were analyzed for enrichment within biological pathways and networks. Two biological networks were highly enriched. Network 1 (Supplementary Figure S1) is associated with inflammatory disease, inflammatory response, organismal injuries and abnormalities (p -value = 10^{-34}). Network 2 (Supplementary Figure S2) is associated with cancer, organismal injury and abnormalities, and reproductive system disease (p -value = 10^{-10}).

4. DISCUSSION

Our previous work found that isoprene-derived SOA induced the expression of oxidative response and inflammatory genes.^{16,21,26,27} Here, we demonstrate that inflammatory and oxidative stress associated miRNAs are also differentially expressed in response to isoprene-derived SOA. There were three major findings from this study: first, miRNAs are likely involved in the modulation of the genomic response to isoprene-derived SOA; second, marked differences exist between the miRNA response to IEPOX-derived SOA and MAE-derived SOA; third, miRNAs may specifically modulate inflammatory response to IEPOX-derived SOA exposure.

The present study suggests that miRNAs are functioning as genomic regulators in relation to SOA in lung cells. There was substantial overlap in the gene targets predicted from the miRNAs differentially expressed in the present study and genes previously identified as differentially expressed in response to isoprene-derived SOA. Of the 52 miRNA–gene target pairings identified in response to IEPOX-derived SOA, 45 of them showed the expected inverse relationship (i.e., miRNA reduced expression, mRNA increased expression) suggestive of miRNA suppression of mRNA expression. On the other hand, all pairings identified in response to MAE-derived SOA demonstrated an upregulation of both miRNA and mRNA, which is not indicative of miRNA suppression. Positive correlation between upregulated miRNA levels and gene expression may be explained by the action of the miRNA blocking a suppressor transcription factor or by an indirect pathway, as has been observed with other miRNAs.^{44,45} Therefore, the current study suggests that miRNAs are involved in the genomic response to SOA and perhaps more extensively involved in the epigenetic regulation of the response to IEPOX-derived SOA than the response to MAE-derived SOA.

The striking differences in miRNA response between the IEPOX- and MAE-derived SOA is consistent with previous studies that show variations in gene expression response based on the specific SOA; however, the exact interplay between miRNAs and gene expression in this context remains to be elucidated fully. Previous studies have shown that MAE-derived SOA induces a gene expression response more robust than that of IEPOX-derived SOA.^{16,21} The present study finds that the miRNA response is more robust in response to IEPOX-derived SOA. In fact, while previous studies have shown specifically inflammatory related genes to be upregulated in response to MAE-derived SOA more than IEPOX-derived SOA, here we find that inflammatory-related miRNAs are downregulated in response to IEPOX-derived SOA, which would lead to upregulation of their respective gene targets. Thus, it is likely that other epigenetic mechanisms are also at play in the control of genomic response to isoprene-derived SOA.

IEPOX-derived SOA responsive miRNAs were found to be associated with inflammatory function and disease. Two biological networks with inflammatory and cancer functions were identified in network analysis of IEPOX-derived SOA responsive miRNAs. The first of these contained insulin as a central node. Increased exposure to air pollution has been linked to insulin resistance in meta-analyses of epidemiologic evidence⁴⁶ as well as in experimental studies.⁴⁷ Additionally, insulin resistance is a known risk factor of asthma.⁴⁸ The second

network contained *TP53*, which represents a potential newly identified pathway not previously found in our gene activation studies of isoprene-derived SOA exposure.^{16,21,27} *TP53* encodes the tumor suppressor p53, which is thought to mediate PM-induced epithelial cell mitochondria-regulated apoptosis, which is relevant to the pathogenesis of lung cancer.⁴⁹ Moreover, *NRF2*-mediated oxidative stress response was an associated pathway of 33 of the target gene-miRNA pairings responsive to IEPOX-derived SOA. *NRF2*, a transcription factor that activates various antioxidant and detoxification enzymes to reduce reactive oxygen species, has been linked in many studies to PM_{2.5} exposure.^{50–53} Notably, *NRF2*-mediated oxidative stress response was shown to be enriched in the gene sets previously found to be responsive to IEPOX- and MAE-derived SOA¹⁶ as well as total isoprene-derived SOA.²⁷ Hence, the networks identified here provide further evidence that the identified miRNAs play a role in IEPOX-derived SOA inflammatory/oxidative stress related epigenetic changes and resultant health outcomes. This is of particular importance to public health because IEPOX-derived SOA has been shown to be the largest contributor to global isoprene-derived SOA, which is up to 30–40% of the total organic material in PM_{2.5}.^{54–56}

While this study provides novel information regarding cellular response to isoprene-derived SOA, it is not without limitations. The use of an immortalized cell line as well as the limitation of delivering PM to submerged cells should be noted. To address these limitations, future studies could explore PM deposition using *in vitro* air-liquid interface technologies or utilize *in vivo* inhalation designs to investigate transcriptional and epigenetic changes induced by isoprene-derived SOA exposure. Additionally, future studies are warranted to assess whether specific miRNAs or a set of miRNAs identified here can act as biomarkers of exposure to isoprene-derived SOA and to evaluate the role of other epigenetic machinery in the genomic response to isoprene-derived SOA.

In summary, in areas such as the southeastern United States, where isoprene emissions are high, understanding the specific cellular responses to isoprene-derived SOA is crucial to public health.^{42,43,54,57} This study provides evidence that miRNAs play a role in isoprene-derived SOA-induced morbidity and do so in a manner that is specific to chemical composition of the SOA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

SOA	secondary organic aerosol
MAE	methacrylic acid epoxide
IEPOX	isomeric isoprene epoxydiols
miRNA	micro-RNA
PM_{2.5}	fine particulate matter (aerosol with aerodynamic diameter <2.5 μm)
KBM	keratinocyte basal medium
KGM	keratinocyte growth medium
LDH	lactate dehydrogenase
PBS	phosphate-buffered saline

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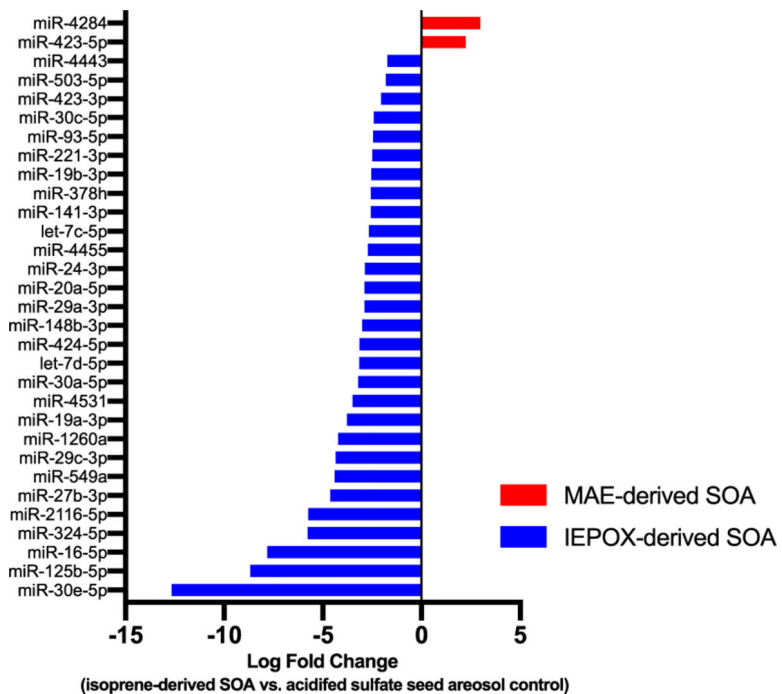


Figure 1. Log fold changes of miRNA expression when exposed to IEPOX- or MAE-derived SOA compared to acidified sulfate aerosol control.

Table 1. Thirty-One miRNAs Identified with Significant Differential Expression Associated with Exposure to Either IEPOX-Derived SOA or MAE-Derived SOA^a

SOA type	miRNA	fold change	FDR-adjusted <i>q</i> -value	<i>p</i> -value	chromosome
IEPOX-derived	<i>miR-30e-5p</i>	-12.67	0.04	0.004	1
	<i>miR-125b-5p</i>	-8.69	0.04	0.003	11
	<i>miR-2116-5p</i>	-5.73	0.04	0.005	15
	<i>miR-549a</i>	-4.41	0.04	0.007	15
	<i>miR-30a-5p</i>	-3.2	0.04	0.007	6
	<i>miR-424-5p</i>	-3.14	0.04	0.002	X
	<i>miR-29a-3p</i>	-2.9	0.04	0.006	7
	<i>miR-30c-5p</i>	-2.42	0.04	0.005	6
	<i>miR-16-5p</i>	-7.82	0.041	0.021	13
	<i>miR-324-5p</i>	-5.78	0.041	0.021	17
	<i>miR-27b-3p</i>	-4.64	0.041	0.011	9
	<i>miR-29c-3p</i>	-4.35	0.041	0.013	1
	<i>miR-1260a</i>	-4.24	0.041	0.013	14
	<i>miR-19a-3p</i>	-3.78	0.041	0.019	13
	<i>miR-4531</i>	-3.49	0.041	0.016	19
	<i>miR-148b-3p</i>	-3.02	0.041	0.021	12
	<i>miR-20a-5p</i>	-2.9	0.041	0.02	13
	<i>miR-24-3p</i>	-2.86	0.041	0.022	9
	<i>miR-4455</i>	-2.73	0.041	0.022	4
	<i>miR-378h</i>	-2.58	0.041	0.011	5
	<i>miR-19b-3p</i>	-2.55	0.041	0.012	13
	<i>miR-423-3p</i>	-2.05	0.041	0.018	17
	<i>miR-503-5p</i>	-1.81	0.041	0.014	X
	<i>miR-4443</i>	-1.73	0.041	0.022	3
	<i>let-7d-5p</i>	-3.16	0.043	0.026	9
	<i>miR-221-3p</i>	-2.5	0.043	0.026	X
	<i>let-7c-5p</i>	-2.67	0.049	0.032	21
	<i>miR-141-3p</i>	-2.58	0.049	0.033	12

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SOA type	miRNA	fold change	FDR-adjusted <i>q</i> -value	<i>p</i> -value	chromosome
	<i>miR-93-5p</i>	-2.47	0.049	0.033	7
MAE-derived	<i>miR-423-5p</i>	2.24	0.03	0.0004	17
	<i>miR-4284</i>	2.99	0.03	0.0009	7

^aSignificance defined as $q < 0.05$.