Air toxics and epigenetic effects: ozone altered microRNAs in the sputum of human subjects

Rebecca C. Fry,1,2,3 Julia E. Rager,1 Rebecca Bauer,2 Elizabeth Sebastian,1 David B. Peden,4,5 Ilona Jaspers,4,5 and Neil E. Alexis4,5
1Department of Environmental Sciences and Engineering, Gillings School of Global Public Health, University of North Carolina, Chapel Hill, North Carolina; 2Curriculum in Toxicology, University of North Carolina, Chapel Hill, North Carolina; 3Center for Environmental Health and Susceptibility, Gillings School of Global Public Health, University of North Carolina, Chapel Hill, North Carolina; 4Center for Environmental Medicine, Asthma, and Lung Biology, School of Medicine, University of North Carolina, Chapel Hill, North Carolina; and 5Department of Pediatrics, University of North Carolina, School of Medicine University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

Submitted 6 December 2013; accepted in final form 21 April 2014

Fry RC, Rager JE, Bauer R, Sebastian E, Peden DB, Jaspers I, Alexis NE. Air toxics and epigenetic effects: ozone altered microRNAs in the sputum of human subjects. Am J Physiol Lung Cell Mol Physiol 306: L1129–L1137, 2014. First published April 25, 2014; doi:10.1152/ajplung.00348.2013.—Ozone (O₃) is a criteria air pollutant that is associated with numerous adverse health effects, including altered respiratory immune responses. Despite its deleterious health effects, possible epigenetic mechanisms underlying O₃-induced health effects remain understudied. MicroRNAs (miRNAs) are epigenetic regulators of genomic response to environmental insults and unstudied in relationship to O₃ inhalation exposure. Our objective was to test whether O₃ inhalation exposure significantly alters miRNA expression profiles within the human bronchial airways. Twenty healthy adult human volunteers were exposed to 0.4 ppm O₃ for 2 h. Induced sputum samples were collected from each subject 48 h preexposure and 6 h postexposure for evaluation of miRNA expression and markers of inflammation in the airways. Genomewide miRNA expression profiles were evaluated by microarray analysis, and in silico predicted mRNA targets of the O₃-responsive miRNAs were identified and validated against previously measured O₃-induced changes in mRNA targets. Biological network analysis was performed on the O₃-associated miRNAs and mRNA targets to reveal potential associated response signaling and functional enrichment. Expression analysis of the sputum samples revealed that O₃ exposure significantly increased the expression levels of 10 miRNAs, namely miR-132, miR-143, miR-145, miR-199a*, miR-199b-5p, miR-222, miR-223, miR-25, miR-424, and miR-582-5p. The miRNAs and their predicted targets were associated with a diverse range of biological functions and disease signatures, noted among them inflammation and immune-related disease. The present study shows that O₃ inhalation exposure disrupts select miRNA expression profiles that are associated with inflammatory and immune response signaling. These findings provide novel insight into epigenetic regulation of responses to O₃ exposure.

inflammation in the airways (4, 22), marked by neutrophil influx. O₃ is also associated with increased risk of asthma as well as increased risk of infection, especially in children and the elderly (27).

There are a variety of mechanisms involved in the cellular response to air pollutant exposure. Animal models have shown that chronic exposure to air pollutants can produce significant changes in gene expression patterns (8, 15, 18, 28, 44), and in humans changes in transcriptional profiles have been correlated with air pollutant exposure (7, 9, 10, 11, 30, 34, 40, 45). Together, these data show that pollutant-induced changes in gene expression levels have biological importance. There is increasing evidence, however, that the epigenome, referring to elements that occur "above the genome," can play a critical role in mediating cellular responses to environmental exposures (20).

MicroRNAs (miRNAs) are key mediators and posttranscriptional modifiers of gene expression. They comprise a large family of small noncoding RNAs that repress or inhibit gene expression by targeting messenger RNA (mRNA) (16, 31). miRNAs can be found in sputum obtained from the central airways (35, 38), a site where O₃ induces a robust inflammatory response and produces changes in immune cell responses following controlled inhalational exposures (5, 17). Although there are known links between miRNA expression changes and air pollutants (19, 37), very few studies have assessed genomewide changes in mRNA expression profiles resulting from air toxicant exposure in vivo. Of the studies that have examined the role of miRNAs in pollutant-induced effects, it appears that pollutants with oxidizing potential have the ability to alter miRNA expression profiles (37). Ozone is a potent oxidizing pollutant and therefore has good potential to alter miRNA expression profiles. We have recently shown that genetics play an important role in determining an individual’s airway inflammatory response to O₃. Specifically, we also showed that the GSTM1 null genotype is a significant modulating factor for the severity and persistence of the airway neutrophil response to O₃ (3). We have also identified distinct genomic signatures in inflammatory responsive and nonresponsive individuals to O₃ (17).

What is less clear, however, is whether epigenetic mechanisms such as miRNAs are modified following O₃ exposure, and in turn what posttranscriptional mRNA targets and biological networks are affected by these changes. In the present study we examine genomewide miRNA expression profiles...
from sputum samples obtained preexposure and postexposure to O₃ in healthy humans and report that O₃ disrupts miRNA expression profiles that are linked to inflammation and immune response function. Moreover, individuals defined as inflammatory responsive to O₃ have a set of constitutively altered miRNAs not seen in inflammatory nonresponsive individuals.

**MATERIALS AND METHODS**

**Ethics statement.** The study protocol was approved by the Institutional Review Board at the University of North Carolina Medical School in Chapel Hill and the US Environmental Protection Agency (EPA), and informed consent was obtained from all subjects before their participation in the study.

**Study subjects.** Twenty healthy nonasthmatic adult subjects aged 18–37 yr, with no history of smoking in the past 10 years, completed the study (Table 1). All subjects underwent a physical examination, a routine blood panel with complete blood cell count, and differential and allergy skin testing for common allergens (cat, cockroach, dust, grass, mite, shellfish, tree, weeds). Subjects were required to have a negative methacholine challenge test result. Female subjects were grass, mite, shellfish, tree, weeds). Subjects were required to have a negative methacholine challenge test result. Female subjects were required to have a negative methacholine challenge test result. Male subjects were required to have a negative urine pregnancy test result before challenge, and all volunteers were required to be free of chronic cardiorespiratory disease and no acute respiratory illnesses within the previous 4–6 wk of O₃ challenge. All subjects had forced expiratory volume in 1 s (FEV₁) and forced vital capacity (FVC) values of 80% or greater of predicted value and FEV₁/FVC ratios of 75% or greater of predicted normal value for height and age. All subjects were screened for their ability to provide an adequate induced sputum sample during their training session 48 h prior to exposure and this sample acted as their preexposure baseline. Pre- and postexposure sputum samples were separated by a minimum of 48 h.

The O₃ exposures were conducted in an exposure chamber at the US EPA Human Studies Facility on the campus of the University of North Carolina (Chapel Hill, NC). Each subject was exposed to 0.4 ppm O₃ for 2 h while performing four 15-min sessions of intermittent moderate exercise (expiratory minute ventilation, 30–40 l/min) on a treadmill, separated by 15 min of seated rest. Lung function, breath sounds, and vital signs were assessed immediately preexposure and postexposure.

Sputum was obtained 6 h after exposure and processed as previously described (3). Sputum samples were used because O₃ preferentially absorbs in the large central airways during exercise, the same region from which sputum samples originate (1). In brief, three 7-min inhalation periods of nebulized hypertonic saline (3, 4, and 5%; UltraNeb 99 ultrasonic nebulizer; DeVilbiss, Jackson, TN) were followed by expectoration of sputum into a sterile specimen cup. Cell-rich “plug” material was selected from the raw sample and treated with a dilute (0.1%) solution of dithiothreitol (Sputolyxin; Calbiochem, San Diego, CA) in Dulbecco PBS. Aliquots of sputum supernatant were collected and immediately frozen and stored at −80°C for future cytokine measurement, and the cell pellet was analyzed for cell viability (with Trypan blue exclusion stain), total cell count, and a portion for differential leukocyte counts (Hema-Stat-3; Fisher Scientific, Hampton, NH). The remaining cells were treated with 1 ml of TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) and frozen at −80°C until RNA extraction. Sputum samples considered acceptable for subsequent processing had a minimum of 75 mg of selected plug material, cell viability greater than 50%, and squamous epithelial cells less than 40%. All sample processing was performed on the same day of collection.

**Sample processing.** Small RNAs were isolated from thawed sputum cell pellet samples. Samples were first disrupted and homogenized in the presence of TRIzol (Invitrogen Life Technologies), and RNA was isolated by the standard TRIzol protocol followed by purification by using the miRNeasy kit (Qiagen) according to the supplier’s instruction. Extracted RNA was quantified with a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA) and its integrity was verified with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA was labeled and hybridized to the Agilent

**Table 1. Characteristics of the study participants and sputum differential cell counts preexposure and postexposure to ozone**

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Age</th>
<th>Sex</th>
<th>Race</th>
<th>BMI</th>
<th>Atpoy</th>
<th>% PMN Pre</th>
<th>% PMN Post</th>
<th>% Change in %PMN</th>
<th>% MAC Pre</th>
<th>% MAC Post</th>
<th>% Eos Pre</th>
<th>% Eos Post</th>
<th>% Lym Pre</th>
<th>% Lym Post</th>
<th>% BEC Pre</th>
<th>% BEC Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28</td>
<td>Female</td>
<td>Caucasian</td>
<td>20.2</td>
<td>0</td>
<td>46.7</td>
<td>66.4</td>
<td>42.1</td>
<td>52</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>Female</td>
<td>Caucasian</td>
<td>20.8</td>
<td>0</td>
<td>15.3</td>
<td>61.8</td>
<td>304.1</td>
<td>81</td>
<td>37</td>
<td>0.4</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>22</td>
<td>Female</td>
<td>Caucasian</td>
<td>29.8</td>
<td>0</td>
<td>10.6</td>
<td>70.2</td>
<td>565.6</td>
<td>88</td>
<td>28</td>
<td>0</td>
<td>0.2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>35</td>
<td>Male</td>
<td>African</td>
<td>29.1</td>
<td>1</td>
<td>78.8</td>
<td>87.7</td>
<td>11.2</td>
<td>19</td>
<td>12</td>
<td>0.6</td>
<td>0.4</td>
<td>1</td>
<td>0.2</td>
<td>0.4</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>18</td>
<td>Male</td>
<td>Caucasian</td>
<td>24.9</td>
<td>1</td>
<td>71</td>
<td>50</td>
<td>29.6</td>
<td>27</td>
<td>48</td>
<td>0.8</td>
<td>0</td>
<td>0.6</td>
<td>0.9</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>22</td>
<td>Female</td>
<td>Caucasian</td>
<td>21.8</td>
<td>1</td>
<td>25.1</td>
<td>68.1</td>
<td>171.3</td>
<td>72</td>
<td>30</td>
<td>0.2</td>
<td>0.3</td>
<td>0.7</td>
<td>0.6</td>
<td>1.6</td>
<td>0.9</td>
</tr>
<tr>
<td>7</td>
<td>20</td>
<td>Male</td>
<td>African</td>
<td>24.6</td>
<td>1</td>
<td>68.8</td>
<td>71.2</td>
<td>3.5</td>
<td>29</td>
<td>27</td>
<td>0</td>
<td>0.9</td>
<td>0.6</td>
<td>0.2</td>
<td>0.4</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>22</td>
<td>Male</td>
<td>Asian</td>
<td>17.8</td>
<td>1</td>
<td>11.6</td>
<td>71.9</td>
<td>521.7</td>
<td>87</td>
<td>22</td>
<td>0</td>
<td>5</td>
<td>0.4</td>
<td>1.4</td>
<td>1.3</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>21</td>
<td>Male</td>
<td>Hispanic</td>
<td>22.5</td>
<td>1</td>
<td>47.9</td>
<td>18</td>
<td>−62.4</td>
<td>44</td>
<td>28</td>
<td>7</td>
<td>51</td>
<td>0.2</td>
<td>0.4</td>
<td>1.3</td>
<td>2.7</td>
</tr>
<tr>
<td>10</td>
<td>24</td>
<td>Female</td>
<td>Caucasian</td>
<td>25.7</td>
<td>0</td>
<td>36.9</td>
<td>64.1</td>
<td>73.7</td>
<td>59</td>
<td>32</td>
<td>0</td>
<td>0</td>
<td>2.2</td>
<td>1.6</td>
<td>1.8</td>
<td>1.9</td>
</tr>
<tr>
<td>11</td>
<td>23</td>
<td>Female</td>
<td>Caucasian</td>
<td>19.2</td>
<td>0</td>
<td>63.8</td>
<td>66.9</td>
<td>4.8</td>
<td>35</td>
<td>32</td>
<td>0.2</td>
<td>0.2</td>
<td>0.4</td>
<td>1.1</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>22</td>
<td>Male</td>
<td>African</td>
<td>25.9</td>
<td>1</td>
<td>54.1</td>
<td>78.4</td>
<td>44.9</td>
<td>37</td>
<td>18</td>
<td>2.7</td>
<td>2.9</td>
<td>0.6</td>
<td>0.2</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>37</td>
<td>Male</td>
<td>Caucasian</td>
<td>27.3</td>
<td>0</td>
<td>30.3</td>
<td>60.4</td>
<td>99.5</td>
<td>67</td>
<td>38</td>
<td>0</td>
<td>2.3</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>37</td>
<td>Female</td>
<td>Caucasian</td>
<td>25.4</td>
<td>0</td>
<td>52.2</td>
<td>71.8</td>
<td>37.5</td>
<td>41</td>
<td>26</td>
<td>0</td>
<td>0.4</td>
<td>0.2</td>
<td>0</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>35</td>
<td>Female</td>
<td>African</td>
<td>25</td>
<td>0</td>
<td>24.3</td>
<td>89.8</td>
<td>270.2</td>
<td>74</td>
<td>10</td>
<td>0.2</td>
<td>0</td>
<td>0.2</td>
<td>0</td>
<td>1.3</td>
<td>0.2</td>
</tr>
<tr>
<td>16</td>
<td>21</td>
<td>Female</td>
<td>Caucasian</td>
<td>27</td>
<td>0</td>
<td>32.8</td>
<td>26.4</td>
<td>−19.5</td>
<td>66</td>
<td>70</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>1.8</td>
<td>0.7</td>
<td>1</td>
</tr>
<tr>
<td>17</td>
<td>29</td>
<td>Female</td>
<td>African</td>
<td>38.3</td>
<td>1</td>
<td>20.5</td>
<td>49.8</td>
<td>142.6</td>
<td>77</td>
<td>47</td>
<td>0.3</td>
<td>0.6</td>
<td>0</td>
<td>0.4</td>
<td>2.6</td>
<td>2.3</td>
</tr>
<tr>
<td>18</td>
<td>29</td>
<td>Female</td>
<td>African</td>
<td>25.2</td>
<td>1</td>
<td>25.1</td>
<td>94</td>
<td>275.1</td>
<td>74</td>
<td>5</td>
<td>1</td>
<td>0.6</td>
<td>0.4</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>33</td>
<td>Male</td>
<td>Caucasian</td>
<td>24.3</td>
<td>1</td>
<td>7.9</td>
<td>73.1</td>
<td>826.2</td>
<td>91</td>
<td>26</td>
<td>0</td>
<td>0.2</td>
<td>0.4</td>
<td>0</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>20</td>
<td>21</td>
<td>Male</td>
<td>Caucasian</td>
<td>23.6</td>
<td>1</td>
<td>19.5</td>
<td>82.4</td>
<td>319.6</td>
<td>77</td>
<td>17</td>
<td>0.9</td>
<td>0.3</td>
<td>1.8</td>
<td>0.3</td>
<td>0.2</td>
<td>0.3</td>
</tr>
</tbody>
</table>
Human miRNA Microarray (v1.0). This microarray assesses the relative expression levels of 534 miRNAs measured with 11,080 probe sets. Microarray results were extracted by using Agilent Feature Extraction software. Microarray data have been submitted to National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) repository (14) and are available under accession number GSE47977.

**Microarray analysis.** Microarray data were normalized by quantile normalization and examined for any batch effects. To eliminate background noise, miRNA probes with signal intensities less than the median signal (signal = 45) across all replicates were eliminated from analysis. Differential expression was defined as a significant difference in miRNA levels between preexposure vs. postexposure to O₃, where four statistical requirements were set, similar to our previous subjects’ sputum samples, a Spearman rank correlation test was used to compare miRNA expression profiles preexposure vs. postexposure by using subject identification as a random effect, similar to microarray analyses performed in previous investigations (32, 39). To control the rate of false positives, q-values were calculated as the minimum “false positive discovery rate” that can occur when identifying significant hypotheses (41). An additional mixed model analysis of covariance was performed to assess the influence of the following potential covariates: age, atopy, body mass index, sex, and race. Inclusion of the possible covariates in the statistical model did not change the results and were therefore excluded from the model used for the final analysis.

**Comparing O₃-responsive miRNAs to immune cell type-specific miRNAs.** Sputum samples contain mixed cell populations, with the predominant cell types in healthy volunteers being macrophages/monocytes and neutrophils (26). To assess whether the O₃-induced changes in miRNA expression profiles were attributable to changes in the distribution of cell populations and hence a confounding factor, the O₃-responsive miRNAs were compared with miRNAs that have been identified as specifically being expressed in certain immune cell populations. Findings from a recent investigation (6) were used in the comparison, where authors performed genomewide miRNA expression profiling for nine human immune cell subsets: neutrophils, eosinophils, monocytes, B cells, natural killer cells, CD4 T cells, CD8 T cells, myeloid dendritic cells, and plasmacytoid dendritic cells (6).

Cell type-specific miRNAs were identified as those specifically expressed on one of the evaluated immune cell types (6). This list of immune cell type-specific miRNAs was compared against the miRNAs identified as O₃ responsive in this study. To test possible relationships between an immune cell-specific miRNA change in expression and changes in immune cell proportions found in the subjects’ sputum samples, a Spearman rank correlation test was performed.

**Predicting targets of O₃-responsive miRNAs.** To understand the impact of O₃-responsive miRNAs on transcript levels, computational predictions of the miRNA targets of the O₃-responsive miRNAs were carried out. The Ingenuity Knowledge Database (Ingenuity Systems, Redwood City, CA) was queried for experimentally observed interactions between miRNAs and their mRNA targets. This database included interactions gathered by Ingenuity Systems curators, as well as interactions from two external databases: TarBase and miRecords. TarBase is a database that represents a comprehensive collection of miRNA targets with experimental support. The interactions in TarBase are curated from both disease- and non-disease-related studies that use methods to increase or decrease the expression of a particular miRNA of interest and evaluate its downstream transcriptional effects (33). miRecords is a database of both experimentally validated miRNA targets gathered through the compilation of findings from 2,705 studies, to date (43). The majority of the information in miRecords is gathered from low-throughput experiments that also involve altering the expression levels of particular miRNAs in animal models (43).

**Comparing mRNA targets to additional O₃-associated transcriptomic databases.** To further substantiate the mRNA targets predicted to be regulated via O₃-responsive miRNAs, the miRNAs were compared against two databases evaluating responses to O₃ exposure at the transcriptional level in humans. Firstly, we compared our predicted mRNA data to data from our recent study (17) where 140 genes were differentially expressed in human volunteers’ sputum samples following O₃ exposure. Secondly, an additional transcriptomic database was obtained by querying the Gene Expression Omnibus (GEO) repository for “ozone exposure humans.” A data series (GSE470) was identified representing transcriptional profiles from bronchial epithelial cells collected from humans preexposure (n = 2) and postexposure (n = 2) to O₃. To analyze the miRNA microarray results, Affymetrix Human Genome U95 (v2) array data were first normalized by robust multichip average via Partek Genomics Suite software. Differential expression was defined as a significant difference in mRNA levels between exposed vs. unexposed samples, where two statistical requirements were set similar to the mRNA analysis: t fold change of ≥1.3 or ≤1.3 (average postexposure/average preexposure); 2) P value < 0.05 (ANOVA). A multiple test correction filter could not be applied in this analysis because of limited sample size. The genes that met these statistical requirements were compared against the list of mRNA targets predicted as regulated by miRNAs.

**Network analysis.** Two biological network analyses were performed to understand the mechanistic responses to O₃ inhalation exposure possibly mediated by miRNA regulation. First, O₃-responsive miRNAs were overlaid onto a global interaction network. Networks containing miRNA signaling were algorithmically constructed based on connectivity, as enabled through Ingenuity Pathway Analysis (Ingenuity Systems). Significance for each constructed network was evaluated by a modified Fisher’s exact test (12). Biological functions and disease signatures within the constructed networks were then identified. Overrepresented functions/diseases were defined as those that contain more targets than expected by chance, as calculated by the modified Fisher’s exact test. A second network analysis was performed, in which the predicted mRNA targets of O₃-responsive miRNAs were assessed. Interaction networks were constructed in a similar manner, and biological functions and disease signatures within the constructed networks were identified.

**In vitro experimentation for qRT-PCR validation.** To validate the miRNA changes identified as associated with O₃ exposure, an in vitro model was used involving differentiated monocyte-derived macrophages. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood obtained from healthy nonsmoking human volunteers via a protocol approved by the University of North Carolina School of Medicine Institutional Review Board for Biomedical Research. The PBMC were isolated by using a Lymphoprep gradient (Life Technologies, Carlsbad, CA). Briefly, the whole blood was diluted with an equal volume of PBS (Life Technologies) and a layer of Lymphoprep was pipetted below the diluted whole blood. The whole blood-Lymphoprep preparation was centrifuged at 800 g for 20 min at room temperature with no break. The PBMC interface was aspirated and washed twice with HBSS containing 2 mM EDTA (Cellgro, Manassas, VA) and 0.1% BSA (Sigma-Aldrich, St. Louis, MO) before isolation of monocytes by negative selection by using the Dynabeads Untouched Human Monocytes Kit according to manufacturer instructions (Life Technologies). The isolated monocytes were plated at 0.5 × 10⁵ cells per well on 12-mm Transwell inserts with 0.4-μm pores (Costar, Cambridge, MA) in RPMI 1640 media supplemented with 10% FBS and 1% penicillin-streptomycin (Life Technologies) and containing 50 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) to induce macrophage differentiation.
had decreases in % PMN after O3 exposure (Table 1). When classified as inflammatory responders and 6 classified as inflammatory nonresponders, where responders were defined as %PMN preexposure − %PMN postexposure ≥12. O3 disrupts mRNA expression profiles in sputum samples. Sputum samples collected preexposure and postexposure were assessed for genomewide changes in miRNA expression profiles by use of the Agilent Human miRNA Microarray. Microarray analysis identified 51 probe sets, representing 10 miRNAs, with significantly increased expression levels following O3 exposure (Fig. 1; see Supplemental Table S1; supplemental material for this article is available online at the Journal website). Altered miRNAs were miR-132, miR-143, miR-145, miR-199a*, miR-199b-5p, miR-222, miR-223, miR-25, miR-424, and miR-582-5p. The data were also analyzed in the context of O3 responders (n = 14) and nonresponders (n = 6). We report that, of the 10 miRNAs that were altered after O3 exposure in the present study, three were significantly different at baseline (e.g., preexposure) when classified according to inflammatory responsiveness. Specifically miR-143, miR-145, and miR-199a* all had lower baseline miRNA expression levels in O3 responders (Fig. 1). Inflammatory responsiveness to O3 (i.e., responder status) had no significant modifying effect on the 10 altered miRNAs after O3 exposure.

Two O3-responsive miRNAs were immune cell type specific. Because most of the human volunteers showed increases in % PMN counts after O3 exposure, it was important to evaluate whether the changes in miRNA expression profiles may be linked to changes in immune cell proportion. Two miRNAs were identified as immune cell type-specific (miR-143-neutrophil specific and miR-223-myeloid cell specific) (6) and were consequently excluded from further analysis since their differential expression levels may have resulted from changes in immune cell populations following O3. Consequently, eight miRNAs were left for further examination.

Interestingly, the O3-associated fold change in expression (log2) for the neutrophil-specific miRNA, miR-143, was significantly correlated with the % change in PMN counts (P = 1.49 × 10^{-8}, R^2 = 0.84) (Fig. 2). This finding suggests that miR-143 is predictive of increases in PMN counts and a potential biomarker for changes in PMNs.
Within the identified miRNA network there was an enrichment for 10 diseases/functions, noted among them inflammation and infection-response (Table 2).

Transcriptional targets of O3-responsive miRNAs were predicted. As mRNA samples were unavailable for the subjects analyzed in the present study, we performed in silico analysis to understand genomic changes likely regulated by the eight O3-responsive miRNAs. Transcriptional targets of the eight O3-responsive miRNAs were predicted by using a database of experimentally observed miRNA-mRNA interactions. Experimental miRNA-mRNA interaction data were not available for one miRNA (miR-582-5p), thus allowing for the final analysis of seven miRNAs. This in silico analysis predicted miR-132 to target 9 mRNAs, miR-145 to target 43 mRNAs, miR-199a* to target 5 mRNAs, miR-199b-5p to target 7 mRNAs, miR-222 to target 21 mRNAs, miR-25 to target 14 mRNAs, and miR-424 to target 178 mRNAs for a total of 277 targets. Since 9 targets overlapped, there were a total of 268 unique mRNA predicted targets (see Supplemental Table S2).

Since seven miRNAs were all increased in expression by O3 exposure, it was predicted that their mRNA targets would show decreased expression. As a form of validation of our predicted analyses, we compared our predicted mRNA data to data from our recent study (17) in which 140 genes were differentially expressed following O3 exposure. A comparison of the 268 predicted mRNA targets reported here with the previously measured 140 genes showed 6 genes to be in common \([\text{CCND1, MYC, retinoblastoma } 1 (\text{RB1}), \text{serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2 (\text{SERPINE2}), superoxide dismutase 2, mitochondrial (\text{SOD2}), and thioredoxin 2 (\text{TXN2})}\] and among them, two changed in the predicted direction (\text{MYC, RB1}).

To further assess the transcriptional targets predicted to be regulated by O3-responsive miRNAs, an additional transcrip-
toxic database was obtained through the GEO repository representing transcriptional profiles from bronchial epithelial cells collected from humans preexposure and postexposure to O$_3$. Statistical analysis from this database identified 452 miRNAs associated with O$_3$ exposure, 18 of which were among those predicted here as regulated via O$_3$-responsive miRNAs (see Supplemental Table S3). Of the 18 miRNAs, 12 were altered by O$_3$ exposure in the direction predicted by miRNA regulation: CBFB (core-binding factor, beta subunit), CCND1, E2F3 (E2F transcription factor 3), HBEGF (heparin-binding EGF-like growth factor), JUN (jun proto-oncogene), MECP2 (methyl CpG binding protein 2), MYC, PNP (purine nucleoside phosphorylase), PP1F (peptidylprolyl isomerase F), SLC7A1 [solute carrier family 7 (cationic amino acid transporter, y+ system), member 1], TMEM109 (transmembrane protein 109), and TMEM251 (transmembrane protein 251). Interestingly, CCND1 and MYC were indeed among those predicted to be at decreased expression levels resulting from O$_3$-induced miRNA alterations in the present study as well as that found in the additional GEO-based transcriptomic analysis.

Network and functional enrichment of O$_3$-responsive miRNA targets. To evaluate the effects of O$_3$-modulated miRNA expression at the systems biology level, networks were constructed with the 268 miRNAs predicted to be regulated by O$_3$-responsive miRNAs. Specifically, 20 significant ($P < 10^{-8}$) networks were constructed that integrated transcriptional targets (see Supplemental Table S4). These transcripts were predicted to have decreased expression levels resulting from upregulated miRNA mediation. Within these networks, an enrichment for various diseases/functions was identified. Of note, the two most significant functions associated with the miRNA target signaling were cell cycle and cell death/survival (Table 3). Three of the constructed networks contained overlapping signaling molecules, and could thus be combined to construct a miRNA-mediated transcript interactome (Fig. 3B).

In vitro validation of O$_3$ altered miRNAs and mRNA targets. To validate that O$_3$ alters the expression of miR-145 and miR199b-5p, human monocyte-derived macrophages were exposed in vitro to 0.4 ppm O$_3$ and air for 2 h. Compared with filtered air, we observed modest increased expression of miR-145 and miR-199b-5p following O$_3$, and consequently robust validation for these miRNAs was not achieved. However, two mRNA targets of these miRNAs, namely CCND1 and MYC, showed statistically significant decreased expression (see Supplemental Fig. S1). Additional time points (6 and 24 h) also demonstrated similar trends for CCND1 and MYC expression (data not shown). These in vitro validation data support the effects of O$_3$-induced expression of targets of miR145 and miR199b-5p at the mRNA level.

**DISCUSSION**

In the present study we set out to investigate the relationship between O$_3$ inhalation exposure and miRNA signaling in human volunteers. To our knowledge, this is the first study to evaluate the effects of O$_3$ exposure on miRNA expression signatures in humans. The majority of subjects responded to a 2-h 0.4 ppm O$_3$ exposure with an expected increase in sputum neutrophils, as well as with changes in immune cell responses previously reported by our group (1, 2, 3, 4, 22, 24). Of the 534 miRNAs examined, we found 10 miRNAs that were significantly altered after O$_3$, and of those eight had known associations with inflammatory signaling (miR-143, miR-145, miR-199a*, miR-199b-5p, miR-222, miR-223, miR-25, miR-424). In particular, miR-223 has been shown to be related to neutrophil hyperactivity (21) and granulocyte development (29), indicating that the airway neutrophil response to O$_3$ could, in part, be influenced by O$_3$-responsive miRNAs within the conducting airways.

We previously demonstrated striking genomic differences after exposure between O$_3$ responders and nonresponders, but surprisingly these genomic differences were not present at preexposure (baseline) (17). Responders did, however, have a primed potential toward PMN influx at baseline because of high preexisting levels of IL-8 (17). Here we demonstrate that, at baseline, O$_3$ responders have lower expression of three miRNAs, namely miR-143, miR-145, and miR-199a*. Interestingly, miR-143 is known to play a role in neutrophil influx (6) and hence may contribute to the constitutively primed airway of responders. These data suggest that interindividual differences in inflammatory responsiveness to O$_3$ may be regulated at the miRNA level.

The cells that are obtained from healthy subjects’ sputum represent a mixed leukocyte population containing mostly macrophages and neutrophils. Thus in this study it was important to identify cell-specific miRNAs that could act as potential confounders since some miRNAs are preferentially expressed in certain cell types (6) and O$_3$ exposure caused a significant increase in neutrophils in most of our subjects. A comparison of the 10 O$_3$-responsive miRNAs to a database of immune cell-specific miRNAs (6) showed that miR-143 and miR-223 are immune cell specific and overabundant in neutrophils and myeloid cells. Interestingly, miR-143 expression changes were significantly correlated with % change in PMN counts, suggesting that miR-143 may serve as a biomarker for increases in PMN cell populations. To eliminate the potential for cell-specific bias, we chose to exclude these two miRNAs from further analyses; however, future studies should likely enrich for particular cell types to minimize this potential difficulty.

To understand the functional consequences of miRNA dysregulation, we performed network and functional enrichment analyses on the eight identified O$_3$-responsive miRNAs. Inflammation and immune function were among the most significantly enriched biological functions associated with these miRNAs. In terms of inflammation, miR-223 has neutrophil-specific associations and miR-145 has links to several physiological features of asthma, a group with known susceptibility to the deleterious effects of O$_3$ exposure. In terms of immune response, miR-199b-5p is a direct regulator of the calcineurin/NFAT pathway (42); miR-132 is associated with interferon-stimulated gene expression (23); and miR-434 and miR-25 are regulators of immune cell differentiation (25). Together, these data suggest that alterations in the expression levels of these miRNAs could be responsible for the immune-related responses we have previously reported at the cell surface marker and cell function level in O$_3$-exposed individuals (2, 3). Moreover, the increased expression of these particular miRNAs along with miRNA-223 reveals new insight into possible acquired immune system mechanisms underlying the well-documented inflammatory response that commonly occurs in humans after O$_3$ inhalation exposure.
process since epithelial cells are a key target cell of O₃’s effect. Ozone exposure may indeed contribute to this effect. Syncytial virus can promote its own survival in airway epithelial cells. Secondary and tertiary by-products generated in the lung lining fluid layer following inhalation.

Table 3. Ten most significant biological functions and disease signatures predicted to be regulated by O₃-responsive miRNAs

<table>
<thead>
<tr>
<th>Biological Function or Disease Signature*</th>
<th>P Value</th>
<th>Number of Inflammation-Associated Transcripts/Total Number of Transcripts</th>
<th>Inflammation-Associated Transcripts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell cycle</td>
<td>1.29E-26</td>
<td>33/81</td>
<td>BCL2, BCL2L11, CCND3, CD44, CDK6, CDKN1A, CDKN1B, ESRI, FGFR2, FGFR1, FO5, FOXO3, HBEFG, HIF1A, HMOX1, HSPA1A/HSPA1B, IKZF1, ITGA5, JUN, MCM2, MMP9, MTR, PP33CA, PTEN, PTGS2, PURA, RB1, RECK, SIRT1, SOD2, VEGFA, WNT3A, WTI</td>
</tr>
<tr>
<td>Cell death and survival</td>
<td>3.99E-26</td>
<td>43/135</td>
<td>ARHGHDIA, BCL2, BCL2L11, BDNF, CARD8, CCND3, CD44, CDK6, CDKN1A, CDKN1B, DDR1, ESRI, FGFR2, FGFR1, FO5, FOXO3, HBEFG, HIF1A, HMOX1, HSPA1A/HSPA1B, ICAM1, IKZF1, ITGA5, JUN, MCL1, MDM2, MMP1, MMP9, MTR, PPP3CA, PTEN, PTGS2, PURA, RB1, RECK, SIRT1, SOD2, TIP3, TIP4, VEGFA, WNT3A, WTI</td>
</tr>
<tr>
<td>Cancer</td>
<td>6.58E-26</td>
<td>46/167</td>
<td>ARHGHDIA, BCL2, BCL2L11, BDNF, CACNA2D1, CCND3, CD44, CDK6, CDKN1A, CDKN1B, DDR1, ESRI, FGFR2, FGFR1, FO5, FOXO3, H3F3A/H3F3B, HBEFG, HIF1A, HMOX1, HSPA1A/HSPA1B, ICAM1, IKZF1, ITGA5, JUN, MCL1, MDM2, MMP1, MMP9, MTR, PPP3CA, PTEN, PTGS2, PURA, RB1, RECK, SIRT1, SOD2, TIP3, TIP4, VEGFA, WNT3A, WTI</td>
</tr>
<tr>
<td>Cellular development</td>
<td>4.16E-25</td>
<td>43/121</td>
<td>ARHGHDIA, BCL2, BCL2L11, BDNF, CCND3, CD44, CDK6, CDKN1A, CDKN1B, DDR1, ESRI, FGFR2, FGFR1, FO5, FOXO3, HBEFG, HIF1A, HMOX1, HSPA1A/HSPA1B, ICAM1, IKZF1, ITGA5, JUN, MCL1, MDM2, MMP1, MMP9, MTR, PPP3CA, PTEN, PTGS2, PURA, RB1, RECK, SIRT1, SOD2, TIP3, TIP4, VEGFA, WNT3A, WTI</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>1.29E-26</td>
<td>33/81</td>
<td>BCL2, BCL2L11, CCND3, CD44, CDK6, CDKN1A, CDKN1B, ESRI, FGFR2, FGFR1, FO5, FOXO3, HBEFG, HIF1A, HMOX1, HSPA1A/HSPA1B, IKZF1, ITGA5, JUN, MDM2, MMP9, MTR, PP33CA, PTEN, PTGS2, PURA, RB1, RECK, SIRT1, SOD2, VEGFA, WNT3A, WTI</td>
</tr>
<tr>
<td>Tumor morphology</td>
<td>4.16E-25</td>
<td>36/72</td>
<td>HIF1A, HMOX1, HSPA1A/HSPA1B, ICAM1, IKZF1, ITGA5, JUN, MCL1, MDM2, MMP1, MMP9, MTR, PPP33CA, PTEN, PTGS2, PURA, RB1, RECK, SIRT1, SOD2, TIMP3, TIP4, VEGFA, WNT3A, WTI</td>
</tr>
<tr>
<td>Cellular growth and proliferation</td>
<td>4.16E-25</td>
<td>43/127</td>
<td>ARHGHDIA, BCL2, BCL2L11, BDNF, CCND3, CD44, CDK6, CDKN1A, CDKN1B, DDR1, ESRI, FGFR2, FGFR1, FO5, FOXO3, HBEFG, HIF1A, HMOX1, HSPA1A/HSPA1B, ICAM1, IKZF1, ITGA5, JUN, MCL1, MDM2, MMP1, MMP9, MTR, PPP33CA, PTEN, PTGS2, PURA, RB1, RECK, SIRT1, SOD2, TIMP3, TIP4, VEGFA, WNT3A, WTI</td>
</tr>
<tr>
<td>Tissue morphology</td>
<td>3.59E-22</td>
<td>42/98</td>
<td>ARHGHDIA, BCL2, BCL2L11, BDNF, CCND3, CD44, CDK6, CDKN1A, CDKN1B, DDR1, ESRI, FGFR2, FGFR1, FO5, FOXO3, HBEFG, HIF1A, HMOX1, HSPA1A/HSPA1B, ICAM1, IKZF1, ITGA5, JUN, MCL1, MDM2, MMP1, MMP9, MTR, PPP33CA, PTEN, PTGS2, PURA, RB1, RECK, SIRT1, SOD2, TIMP3, TIP4, VEGFA, WNT3A, WTI</td>
</tr>
<tr>
<td>Organismal survival</td>
<td>3.97E-22</td>
<td>42/109</td>
<td>HIF1A, HMOX1, HSPA1A/HSPA1B, ICAM1, IKZF1, ITGA5, JUN, MCL1, MDM2, MMP1, MMP9, MTR, PPP33CA, PTEN, PTGS2, PURA, RB1, RECK, SIRT1, SLCA7A1, SOD2, TIP4, TIP5, VEGFA, WNT3A, WTI</td>
</tr>
<tr>
<td>Hematological system development and function</td>
<td>1.21E-19</td>
<td>42/93</td>
<td>ARHGHDIA, BCL2, BCL2L11, BDNF, CCND3, CD44, CDK6, CDKN1A, CDKN1B, DDR1, ESRI, FGFR2, FGFR1, FO5, FOXO3, HBEFG, HIF1A, HMOX1, HSPA1A/HSPA1B, ICAM1, IKZF1, ITGA5, JUN, MCL1, MDM2, MMP1, MMP9, MTR, PPP33CA, PTEN, PTGS2, PURA, RB1, RECK, SIRT1, SLCA7A1, SOD2, TIP4, TIP5, VEGFA, WNT3A, WTI</td>
</tr>
<tr>
<td>Cell morphology</td>
<td>1.55E-19</td>
<td>40/102</td>
<td>ARHGHDIA, BCL2, BCL2L11, BDNF, CCND3, CD44, CDK6, CDKN1A, CDKN1B, DDR1, ESRI, FGFR2, FGFR1, FO5, FOXO3, HBEFG, HIF1A, HMOX1, HSPA1A/HSPA1B, ICAM1, IKZF1, ITGA5, JUN, MCL1, MDM2, MMP1, MMP9, MTR, PPP33CA, PTEN, PTGS2, PURA, RB1, RECK, SIRT1, SLCA7A1, SOD2, TIMP3, TIP4, VEGFA, WNT3A, WTI</td>
</tr>
</tbody>
</table>

*Based on 268 mRNA predicted targets from 7 O₃-responsive miRNAs.

In silico predictive analysis was used to determine which transcriptional targets would likely be impacted by the observed changes in O₃-responsive miRNAs where only experimentally verified interactions were used. We found that many of the transcriptional targets play a role in cell cycle control with known links to airway disease. Altered cell cycle control may be an advantageous mechanism by which human respiratory syncytial virus can promote its own survival in airway epithelial cells. Ozone exposure may indeed contribute to this process since epithelial cells are a key target cell of O₃’s secondary and tertiary by-products generated in the lung lining fluid layer following inhalation.

As a form of validation of these predicted results, we utilized and compared data from our recent study in 27 adult volunteers that looked at mRNA changes in sputum 5 h following 0.4 ppm O₃ exposure. That study showed differential expression in 140 genes (17). We report that there were six genes in common with our predicted mRNA in this study and previously measured mRNA, including MYC and RB1, both of which had changes in the predicted direction. Although the changes in the present study are based on in silico analysis, which contain certain inherent limitations, these validation data give us increased confidence that the altered miRNAs reported here indeed impact the expression of transcripts following exposure to O₃.
In the present study, healthy individuals were used to investigate the effects of O₃ exposure on miRNA levels. Within these individuals there were demonstrated differences in the magnitude of their inflammatory response to O₃. These differences were associated with differences in miRNA expression at baseline. These data suggest that miRNAs may influence interindividual susceptibility to O₃ and support inclusion of miRNA evaluation as well as other known genetic risk factors such as the GSTM1 null genotype (3), as necessary risk factors to measure to understand the effects of O₃ on susceptible populations.

Conclusions. This study demonstrates that O₃ exposure is associated with altered miRNA expression profiles within the human respiratory tract. The identified O₃-responsive miRNAs and their targets are notable for their involvement in inflammation and immune cell response. The present study also highlights the role of miRNAs as predictors of interindividual variability in the inflammatory response to O₃.

ACKNOWLEDGMENTS

The authors acknowledge Heather Wells, Danuta Sujkowska, Lynn Newlin-Clapp, Carol Robinette, Fernando Dimeo, Aline Kalla, Martha Almond, Wenli Zhang, and Margaret Herbst for laboratory and clinical assistance.

GRANTS

This research was supported in part by NIH grants ES-012796, ES-018417, ES-010126, AT-002620, U19-AI077437, ES019315, ES007018, and EPA Cooperative Agreement CR83346301. Disclaimer: Although the research described in this article has been funded wholly or in part by the United States EPA through cooperative agreement CR833463-01 with the Center for Environmental Medicine, Asthma, and Lung Biology at the University of North Carolina at Chapel Hill, it has not been subjected to the Agency's required peer review and therefore does not necessarily reflect the views of the Agency and no official endorsement should be inferred. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES