

# MicroRNAs as modulators of smoking-induced gene expression changes in human airway epithelium

Frank Schembri<sup>a,1</sup>, Sriram Sridhar<sup>b,1</sup>, Catalina Perdomo<sup>c</sup>, Adam M. Gustafson<sup>d</sup>, Xiaoling Zhang<sup>d</sup>, Ayla Ergun<sup>e</sup>, Jining Lu<sup>a</sup>, Gang Liu<sup>a</sup>, Xiaohui Zhang<sup>a</sup>, Jessica Bowers<sup>f</sup>, Cyrus Vaziri<sup>b</sup>, Kristen Ott<sup>c</sup>, Kelly Sensinger<sup>f</sup>, James J. Collins<sup>e</sup>, Jerome S. Brody<sup>a</sup>, Robert Getts<sup>f</sup>, Marc E. Lenburg<sup>a,b,d</sup>, and Avrum Spira<sup>a,b,d,2</sup>

<sup>a</sup>Pulmonary Center, <sup>b</sup>Department of Pathology and Laboratory Medicine, and <sup>c</sup>Genetics and Genomics Graduate Program, Boston University School of Medicine, Boston, MA 02118; <sup>d</sup>Bioinformatics Program, Boston University School of Engineering, Boston, MA 02215; <sup>e</sup>Department of Biomedical Engineering and Center for BioDynamics and Howard Hughes Medical Institute, Boston University, Boston, MA 02215; and <sup>f</sup>Genisphere, Inc., Hatfield, PA 19440

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We have shown that smoking impacts bronchial airway gene expression and that heterogeneity in this response associates with smoking-related disease risk. In this study, we sought to determine whether microRNAs (miRNAs) play a role in regulating the airway gene expression response to smoking. We examined whole-genome miRNA and mRNA expression in bronchial airway epithelium from current and never smokers ( $n = 20$ ) and found 28 miRNAs to be differentially expressed ( $P < 0.05$ ) with the majority being down-regulated in smokers. We further identified a number of mRNAs whose expression level is highly inversely correlated with miRNA expression *in vivo*. Many of these mRNAs contain potential binding sites for the differentially expressed miRNAs in their 3'-untranslated region (UTR) and are themselves affected by smoking. We found that either increasing or decreasing the levels of mir-218 (a miRNA that is strongly affected by smoking) in both primary bronchial epithelial cells and H1299 cells was sufficient to cause a corresponding decrease or increase in the expression of predicted mir-218 mRNA targets, respectively. Further, mir-218 expression is reduced in primary bronchial epithelium exposed to cigarette smoke condensate (CSC), and alteration of mir-218 levels in these cells diminishes the induction of the predicted mir-218 target MAFG in response to CSC. These data indicate that mir-218 levels modulate the airway epithelial gene expression response to cigarette smoke and support a role for miRNAs in regulating host response to environmental toxins.

cigarette smoke | mir-218 | bronchial airway epithelium

Approximately 1.3 billion people smoke cigarettes worldwide, which contributes to 5 million preventable deaths per year (1). Smoking is a significant risk factor for lung cancer (2), the leading cause of cancer-related death in the United States, and chronic obstructive pulmonary disease (COPD), the fourth leading cause of death worldwide. Cigarette smoking has been found to induce a number of genetic and molecular changes in the respiratory tract, including cellular atypia (3), loss of heterozygosity (4, 5), and promoter hypermethylation (6), which can occur in cytologically normal airway epithelium (5, 7). We have characterized the effects of smoking on the human airway epithelial transcriptome and found that smoking induces expression of airway genes involved in regulation of oxidant stress, xenobiotic metabolism, and oncogenesis while suppressing those involved in regulation of inflammation and tumor suppression (8). In addition, we have described a pattern of gene expression in cytologically normal large airway epithelial cells that can serve as a biomarker for lung cancer elsewhere in the lung (9). These studies suggest that gene expression changes in airway epithelium reflect host response to and damage from cigarette smoke; however, the regulatory mechanisms mediating these gene expression changes are unclear.

MicroRNAs (miRNA) are small noncoding RNAs that serve to down-regulate gene expression by suppression of translation or messenger RNA (mRNA) degradation. Thirty percent of protein-coding genes are predicted to be regulated by miRNAs (10).

Hundreds of human miRNAs have been cloned (11), with each of them predicted to regulate hundreds of genes by complementary binding to the 3'-untranslated region (UTR) of target mRNAs (12). A number of miRNAs have been shown to play important roles in developmental timing, cell death, carcinogenesis, and stress responses *in vitro* (13–16), such as hypoxia (17). Whereas miRNA expression has been examined in lung tumors (18–24), neither the expression of miRNA in bronchial epithelium nor the effect of smoking on bronchial airway miRNA expression has been characterized, and it is unclear what role miRNAs play in regulating the physiological response to environmental exposures such as tobacco smoke.

In this study, we have profiled the miRNAs expressed in a relatively pure population of bronchial airway epithelial cells and identified those that are differentially expressed with smoking. Additionally, by whole-genome profiling of both miRNA and mRNA from the same *in vivo* samples, we have identified mRNAs that are potentially regulated by smoking-induced changes in miRNA expression. Through *in vitro* transfection experiments, we have shown that modulating the expression of one such miRNA (mir-218) is sufficient to alter the expression of a subset of the mRNAs that are both predicted targets of this miRNA and altered by smoking *in vivo*. Further, we have shown that cigarette smoke condensate (CSC) suppresses the expression of mir-218 in primary bronchial epithelial cells and that altering the levels of mir-218 in these cells attenuates the CSC-dependent induction of mir-218 target mRNAs. These studies suggest that smoking-dependent changes in miRNA expression levels mediate some of the smoking-induced gene expression changes in airway epithelium and that miRNAs might therefore play a role in the host response to environmental exposures and the pathogenesis of smoking-related lung disease.

## Results

**Smoking Affects Expression of miRNAs in Bronchial Epithelium.** MicroRNA and mRNA expression levels in bronchial epithelial RNA collected from 20 volunteers (10 current smokers, 10 never smokers) recruited for this study were determined by using microarrays [supporting information (SI) Fig. S1]. Demographic data for these subjects are presented in Table 1. After miRNA microarray data preprocessing (Fig. S2A), 232 miRNAs were detected above back-

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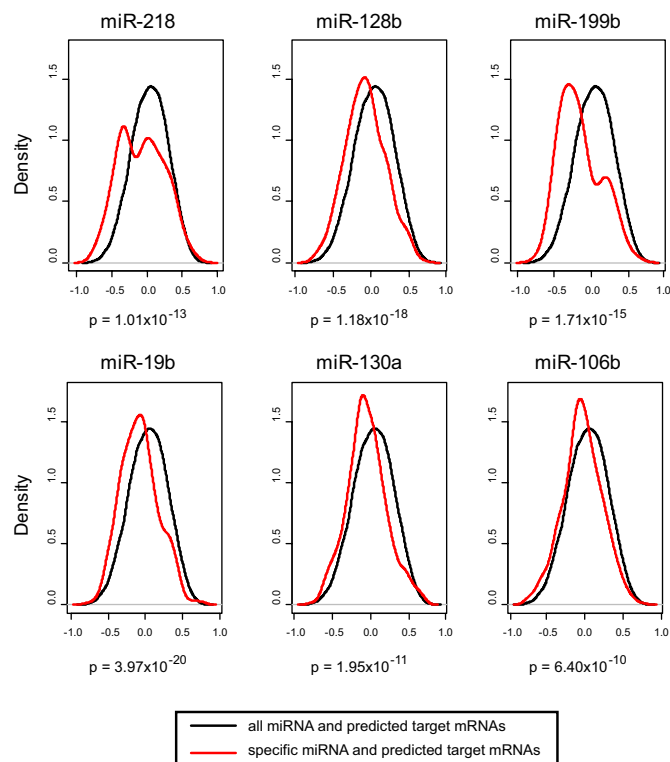
<sup>1</sup>F.S. and S.S. contributed equally to this work.

<sup>2</sup>To whom correspondence should be addressed at: Boston University, 715 Albany Street, R304, Boston, MA 02118. E-mail: [aspira@bu.edu](mailto:aspira@bu.edu).

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**Fig. 3.** Relationship between the expression of miRNAs and their predicted mRNA targets in airway epithelium of current and never smokers. The distribution of Pearson correlation coefficients calculated between the miRNA and predicted target mRNA expression for 6 of the 28 differentially expressed miRNAs is shown in red. All 232 miRNAs and their predicted targets are in black. There is a significant increase in the degree of negative correlation between differentially expressed miRNAs and their targets relative to all miRNAs and their targets, which reflects the inhibitory effects of miRNAs on target gene expression. These 6 miRNAs have the highest number of predicted mRNA targets with significantly anticorrelated expression in vivo.

genes are involved in cell–cell adhesion, cellular signaling, and cytoskeletal structure (Table S3).

**Regulation of mir-218 in Response to Acute in Vitro Cigarette Smoke Exposure.** To further investigate whether cigarette smoke is responsible for the miRNA expression differences seen in airway epithelial cells from smokers and nonsmokers, we performed 2 experiments. First, a publicly available mRNA microarray expression dataset (GSE10700) of normal human bronchial epithelial (NHBE) cells exposed to cigarette smoke was analyzed using Gene Set Enrichment Analysis (GSEA) (26). We found that there was significant overrepresentation of mir-218 targets among the genes most up-regulated by cigarette smoke exposure (SI Methods, Table S4). This indicates that the targets of mir-218 are induced upon acute exposure to cigarette smoke and suggests that mir-218 expression likely decreases upon such exposure. To confirm this, we next exposed NHBE cells to 20  $\mu\text{g/ml}$  CSC. The concentration of CSC used was similar to that in other studies that exposed NHBE cells to CSC (27, 28). We found an almost 2-fold reduction in mir-218 levels in cells exposed to CSC at 24 h relative to DMSO-treated controls (Fig. 2B). Thymidine incorporation assays confirmed that the 20  $\mu\text{g/ml}$  CSC dose used does not inhibit cell proliferation or DNA synthesis in NHBE cells (data not shown), suggesting that the mir-218 changes observed are unlikely due to growth inhibition or toxicity.

**Overexpressing mir-218 in Vitro Preferentially Suppresses Its Predicted Targets.** To explore the functional significance of the inverse correlation between the expression of miRNAs and their predicted

targets, we performed a series of in vitro transfection experiments (see Fig. S2C). First, we increased mir-218 levels in H1299 cells by transient transfection with a synthetic RNA oligonucleotide mimic of mir-218 precursor (premir-218) or a scrambled sequence that served as a negative control. After confirming that this resulted in increased levels of mature mir-218 by real-time PCR (data not shown), Affymetrix all-exon microarrays were run to examine mRNA expression in these transfectants relative to the negative control. GSEA showed a significant enrichment of predicted mir-218 targets among the genes most down-regulated in H1299 cells transfected with mir-218 ( $P < 0.01$ ), confirming that overexpression of mir-218 results in the down-regulation of many of its predicted targets. GSEA was then performed to determine whether any literature-defined pathways are altered in response to increased mir-218 levels in vitro. Several groups of genes involved in various cell cycle pathways and the p53, apoptosis, and Rho/Rac/Ras pathways (false discovery rate, FDR  $< 0.17$ ) were enriched. Targets of mir-218 involved in apoptosis pathways were also anticorrelated to its expression in vivo (Table S3), suggesting that mir-218 might either directly or indirectly affect genes involved in these processes.

A Welch's  $t$  test yielded 1235 genes that changed with mir-218 overexpression ( $P < 0.05$ ) relative to the negative control transfectants, with the large majority ( $\approx 83\%$ ) being down-regulated by elevated mir-218 levels. Of these 1235 genes, 85 were database-predicted targets of mir-218. A  $\chi^2$  test confirmed ( $P = 0.0001$ ) that predicted targets of mir-218 were more likely to be differentially expressed with its overexpression compared with nontargets. Almost all ( $\approx 95\%$ ) of these 85 predicted target genes were down-regulated with overexpression of mir-218 (Fig. 4), indicating that among the genes that are altered by increasing mir-218 levels, its predicted targets are more likely to have lower expression relative to controls than nontargets (Fisher's exact test,  $P = 0.002$ ).

We next sought to determine the relationship between genes that have altered expression in response to elevated mir-218 in vitro and the genes that are associated either with mir-218 levels or with smoking in vivo. For this analysis, we examined the in vivo expression patterns of the 85 putative mir-218 targets that are altered in response to elevated mir-218 levels in vitro. The expression of 21 of these 85 genes is highly inversely correlated (Pearson correlation,  $P < 0.1$ ) with mir-218 levels in vivo (see Fig. 4). This is significant (chi-square  $P = 0.01$ ) as expression of only 16% of the predicted mir-218 targets examined in vivo is significantly inversely correlated with mir-218 expression. Five of these 21 inversely correlated genes are differentially expressed in smokers ( $P < 0.1$ ) and each of the 5 shows significantly lower expression ( $P < 0.05$ ) in the presence of elevated mir-218 in vitro and higher expression in smokers (where mir-218 expression is repressed). These 5 genes (MAFG, ECOP, LASP1, NAC-1, and NFE2L1) thus are candidates for being regulated by changing mir-218 levels in response to cigarette smoke. Using qRT-PCR, we validated the effects of overexpressing mir-218 on the levels of these 5 genes in both H1299 and primary NHBE cells. We found that increasing mir-218 levels in H1299 cells caused significant decreases in all 5 genes, but in NHBE cells only 3 (ECOP, NAC-1, and LASP1) of the 5 genes were significantly repressed (Fig. S5A and Fig. S6A). The remaining two targets (MAFG, NFE2L1) are expressed at very low levels at baseline in NHBE cells compared with H1299 cells.

#### Knockdown of mir-218 Increases Expression of mir-218 Target Genes.

In the second experiment to determine whether mir-218 might regulate the expression of smoking-related genes, we sought to determine whether the endogenous levels of mir-218 serve to repress the expression of mir-218-targeted mRNAs. To test this hypothesis, we decreased the level of mir-218 in both H1299 and NHBE cells by transfection of an antisense oligonucleotide to mir-218 (anti-mir-218), using a scrambled-sequence oligo as a control. After finding that transfection with anti-mir-218 resulted in a 4-fold reduction in mir-218 levels in H1299 cells and a 10-fold



type (32). This may be because miRNA levels are tightly linked to degree of cellular differentiation and that reduced expression of miRNAs in cancer is associated with more dedifferentiated tumors. Consistent with this model, the down-regulation of miRNA expression in airway epithelial cells with smoking may be a consequence of repetitive airway injury that results in accumulation of proliferating and dedifferentiated cells.

Several studies have sought to establish regulatory relationships between miRNA and mRNA expression (33–36). By collecting miRNA and mRNA from the same samples, we were able to discover correlations between miRNA and mRNA expression profiles both across patient groups and within individuals. Whereas this represents the first study characterizing the effects of smoking on miRNA expression, several of the miRNAs from our analyses have been implicated in other diseases. Mir-218, which was down-regulated in smokers and by CSC exposure *in vitro*, is transcribed within the intronic region of the known tumor suppressor gene *SLIT2* (11). *SLIT2*, which is frequently inactivated in lung and breast tumors (37), was also significantly down-regulated in smokers with expression correlating to that of mir-218 in our *in vivo* samples. A second identical copy of mir-218 occurs within the *SLIT3* locus, and expression of *SLIT3* has also been shown to be down-regulated in lung cancer (18). Mir-218 levels have also been found to be decreased in cervical carcinoma cells (38), suggesting a potential general role of mir-218 as a tumor suppressor. Three additional miRNAs (mir-15a, mir-199b, and mir-125b) that are down-regulated in smokers have also been shown to be down-regulated in other cancers including chronic lymphocytic lymphoma (CLL) (39), lung (18), and breast cancer (40). These findings suggest that the down-regulation of miRNAs in smokers could be related to the development of tobacco-related cancers. Given the ability of gene expression differences in the airway to serve as a biomarker for the presence of lung cancer elsewhere in the respiratory tract (9), our finding that miRNAs implicated in carcinogenesis are differentially expressed in the airway epithelium of smokers suggests that airway miRNA expression could potentially serve as an indicator of smoking-induced disease processes.

Functional analysis of the predicted targets of mir-218 that are anticorrelated with its expression *in vivo* reveals an overrepresentation of genes involved in cell structure, cell–cell adhesion, and cell signaling and ion transport pathways. A subset of these targets is also altered with smoking. These include transcriptional regulators such as MAFG and NAC-1, signaling genes such as ECOP (NF $\kappa$ B pathway), and genes involved in cellular structure (LASP1). A predicted mir-218 target of particular interest is the transcription factor MAFG, which has been shown previously (8), as well as in this study to be up-regulated in the bronchial epithelium of smokers and on exposure of cells to CSC *in vitro*. Binding sites for MAFG are overrepresented among genes that are differentially expressed in the epithelial cells of smokers, suggesting that alteration of mir-218 levels could indirectly influence the expression of MAFG targets.

The argument for the smoking-dependent regulation of mir-218 contributing to the regulation of smoking-dependent changes in mRNA expression rests on our *in vitro* perturbation experiments. In H1299 and NHBE cell lines, transient transfections both increasing and decreasing mir-218 levels lead to decreased and increased target expression, respectively, suggesting that altered levels of this miRNA are sufficient to regulate expression of these genes. Furthermore, blocking the CSC-dependent down-regulation of mir-218 by overexpressing mir-218 attenuates the CSC-dependent increase in MAFG expression. A similar, but less dramatic attenuation of the CSC-dependent increase in MAFG expression is observed when mir-218 levels are knocked down. These data suggest that the smoking-dependent modulation of mir-218 contributes to the regulation of mir-218 targets. However, because CSC still has an appreciable effect on MAFG expression even when mir-218 levels are experimentally manipulated, it is likely that other mechanisms contribute to the regulation of smoking-modulated gene expression.

Although we were able to identify miRNAs that are differentially expressed in smokers and correlate with mRNA changes from the same individuals, the small sample size of our study may have limited our power to detect additional smoking-related miRNA and/or mRNA changes. Whereas differentially expressed miRNAs tended to be down-regulated in smokers, we did not explore the mechanisms by which miRNAs are down-regulated. We were able to identify significant levels of anticorrelated expression between miRNAs that are affected by smoking and their target mRNAs, suggesting that miRNAs are modulating mRNA expression in response to smoking. We were further able to establish a role for the levels of mir-218 in regulating the expression of its predicted targets *in vitro*, using H1299 and NHBE cells. Whereas increasing mir-218 levels in these cells led to significant decreases in expression of many of its targets, down-regulating mir-218 in these cells led to only modest increases in target expression. One possible reason for this disparity is that we were unable to completely abrogate expression of mir-218, and the modest changes could have been a result of residual levels of mir-218 in these cells.

In summary, we have profiled global miRNA expression in the bronchial epithelium of healthy current and never smokers. Our findings suggest that smoking causes many miRNAs to be down-regulated in these epithelial cells. Messenger RNA expression profiles from the same individuals show that expression of putative targets of these miRNAs is generally up-regulated in the airway epithelial cells of smokers. We have further demonstrated that modulating levels of mir-218 *in vitro* alters the expression of its predicted targets and attenuates the smoking-dependent induction of target mRNA expression. These findings suggest that miRNAs may play a role in regulating the gene expression response to tobacco exposure in airway epithelial cells. MicroRNA profiles obtained from these cells might therefore serve as biomarkers for smoking-related lung diseases and help elucidate the regulatory mechanisms that mediate the host response to tobacco smoke exposure and other environmental agents.

## Methods

**Study Population.** We recruited never ( $n = 10$ ) and current ( $n = 10$ ) smoking volunteers to undergo fiberoptic bronchoscopy at Boston Medical Center. Subjects with significant second-hand environmental cigarette exposure, respiratory symptoms, or regular use of inhaled medications were excluded. For participants, a detailed smoking history was obtained, including cumulative tobacco exposure (measured in pack years), age when they began smoking, and second-hand tobacco exposure. All individuals were screened with routine chest x-ray and spirometry and were excluded if they had evidence of pulmonary pathology. Temporal (3 months) and technical replicates were collected from 2 participants. The study was approved by the Institutional Review Board of Boston Medical Center and all participants provided written informed consent.

**Sample Collection.** Bronchial airway epithelial cells were obtained from bronchial brushings of the right mainstem bronchus taken during fiberoptic bronchoscopy with an endoscopic cytobrush (Cellebriety Endoscopic Cytobrush, Boston Scientific). The epithelial cell content of the bronchial brushings performed in our clinic is routinely greater than 90% (8). The brushes were immediately placed in RNeasy Protect Cell Reagent (QIAGEN) and stored at 4°C until RNA was isolated and fractionated into low molecular weight (LMW) and high molecular weight (HMW) fractions, by using the miRNeasy mini kit (QIAGEN) (see *SI Methods*).

**MicroRNA Microarray Hybridization and Data Analysis.** Three hundred nanograms of LMW RNA were labeled using the FlashTag labeling kit (Genisphere, Inc.), according to manufacturer's protocol (see *SI Methods*). After labeling, samples were incorporated into a hybridization mix that was then applied to Invitrogen NCode miRNA microarrays containing 1,053 miRNAs from 6 species (467 human miRNAs) printed in triplicate. The arrays were scanned on a GenePix 4000 scanner (Axon/Molecular Devices).

Raw data were extracted by using GenePix Pro 4.0 (Molecular Devices), quantile normalized, and log transformed using the limma package for R (<http://www.r-project.org/>). On the basis of Principal Component Analysis, 1 nonsmoker sample was removed from further analysis. In addition to our sample filter, only miRNA replicates that were detected at levels 2 standard deviations above the average background in at least 6 samples were considered for further analysis.

The median of the remaining replicates for each miRNA was then taken to obtain a single expression value for each miRNA, leaving a total of 232 distinct miRNAs that were analyzed further.

**mRNA Microarray Hybridization and Data Analysis.** One microgram of the HMW RNA fraction from each sample was used as starting material for hybridization to Human Exon 1.0 ST GeneChips as described (41). Transcript-level expression estimates for the  $\approx 17,800$  empirically supported transcripts were used for our analysis (see *SI Methods*).

**Correlating miRNA and mRNA Expression.** Pearson correlations [and associated *P* values] were calculated for all miRNA–mRNA pairs across all samples, and the Benjamini-Hochberg false discovery correction (42) was used to adjust for multiple comparisons. Pairs of miRNA–mRNA with statistically significant inverse correlations were then cross-referenced with two target prediction databases [PicTar (43) or TargetScan, version 3.1 (10)] to identify those where the mRNA is a predicted target of the miRNA.

**Validating Potential mRNA–miRNA Regulatory Relationships via Transient Transfection of miRNA in H1299 and NHBE Cell Lines.** H1299 cells (an immortalized human lung carcinoma cell line) and primary human bronchial epithelial cells (NHBE, Cell Applications) were cultured in 6-well plates for 24 hours prior to transfection (see *SI Methods*). MicroRNA-218 was overexpressed or knocked down using synthetic oligonucleotides at a concentration of 30 nM with the appropriate negative controls ( $n = 5$ –6 per group). Cells were harvested at 48 hours posttransfection, miRNA/mRNA were isolated and run on Affymetrix Exon arrays (H1299 overexpression group only) and qRT-PCR (all 4 groups).

**Cigarette Smoke Exposure in NHBE Cells.** We used a commercially available cigarette smoke condensate (Murty Pharmaceuticals) prepared by smoking University of Kentucky 1R3F research cigarettes on a standard Federal Trade Commission smoking machine. NHBE cells were plated in 6-well plates as described. Cigarette smoke condensate was diluted to 20  $\mu\text{g/ml}$  in growth media and applied to the NHBE cells ( $n = 6$ ), with DMSO-treated (1  $\mu\text{g/ml}$ ,  $n = 6$ ) and untreated ( $n = 6$ ) cells serving as controls. The potential toxicity of this concen-

tration of CSC was evaluated using [ $^3\text{H}$ ]thymidine incorporation assays (see *SI Methods*). Cells were harvested 24-h postexposure and RNA was isolated from these cells by using the miRNeasy mini kit according to manufacturer's protocol.

**Measuring the Effect of CSC on NHBE Cells with Altered mir-218 Levels.** NHBE cells were plated as described in 6-well plates. Cells were first transfected with either premiR-218 or anti-mir-218 along with the appropriate Cy-3-labeled scrambled oligo ( $n = 6$  in each group), using the PrimeFect reagent. After confirming  $\approx 90\%$  transfection efficiency at 24 h, the transfection media were removed and cigarette smoke condensate was applied to cells at a concentration of 20  $\mu\text{g/ml}$ . The cells were then lysed at 24 h post-CSC exposure and RNA was isolated as described above.

**Statistical Analysis of Gene Expression Data.** Differential expression of miRNAs and mRNAs was determined using a Welch's *t* test. GSEA (26) was performed to determine whether sets of genes comprising either various literature-derived pathways or the sequence-predicted targets of mir-218 targets are perturbed when mir-218 levels are altered in vitro. Hierarchical clustering was performed on z-score-normalized data, using CLUSTER and TREEVIEW software.

**Real-Time PCR Validation.** Quantitative real-time PCR was used to confirm the differential expression of a select number of miRNAs in vivo and target mRNAs in vitro (see *SI Methods*).

**Additional Information.** Additional information including raw expression data from microarrays run in vivo and results from correlation analysis can be obtained on the website accompanying this article (<http://pulm.bumc.bu.edu/cgi-bin/mirDB/home.cgi>). All mRNA and microRNA microarray data have also been deposited in the GEO database under accession number GSE14224.

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