NF-κB-Mediated Inflammation Leading to EMT via miR-200c Is Involved in Cell Transformation Induced By Cigarette Smoke Extract

Yue Zhao,*††††1 Yuan Xu,*††††1 Yuan Li,*†††† Wenchao Xu,*†††† Fei Luo,*†††† Bairu Wang,*†††† Ying Pang,*†††† Quanyong Xiang,‡††† Jianwei Zhou,† Xinru Wang,†† and Qizhan Liu*1††2

*Institute of Toxicology and †The Key Laboratory of Modern Toxicology, Ministry of Education, School of Public Health, Nanjing Medical University, Nanjing 210029, Jiangsu, P. R. China; ‡Jiangsu Center for Disease Control and Prevention, Nanjing 210029, Jiangsu, P. R. China

1These authors contributed equally to this study.

2To whom correspondence should be addressed at Institute of Toxicology, School of Public Health, Nanjing Medical University, 818 East Tianyuan Road, Jiangning District, Nanjing 210029, Jiangsu, P. R. China. Fax: +86-25-8652-7613. E-mail: drqzliu@hotmail.com.

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Cigarette smoking constitutes a major human health hazard because it is the most important risk factor for lung cancer. Although evidence for smoking-induced lung cancer in humans is strong, the molecular mechanisms by which smoking causes cancer remain to be established. In this investigation, we evaluated the roles of inflammation and the epithelial-mesenchymal transition (EMT) in cigarette smoke extract (CSE)–induced transformation of human bronchial epithelial (HBE) cells. The results showed that chronic exposure to CSE induced EMT and transformation of these cells. Activation of nuclear factor-κB (NF-κB) by CSE increased levels of the proinflammatory interleukin-6 (IL-6), and acute and chronic exposures to CSE caused decreases in miR-200c levels. By blocking NF-κB with Bay11-7082 and IL-6 with anti-IL-6 antibody and enhancement of IL-6 with human recombinant IL-6, we found that the NF-κB signal pathway was involved in CSE-induced increases of IL-6, which suppressed miR-200c expression and promoted EMT. Moreover, IL-6 was necessary for maintenance of CSE-induced transformation and for malignant progression of HBE cells. Finally, blocking of NF-κB with Bay11-7082 prevented CSE-induced EMT and malignant transformation due to decreases of E-cadherin and miR-200c and elevations of IL-6, N-cadherin, and vimentin. Thus, we have defined a link between inflammation and EMT, processes involved in the malignant transformation of cells caused by CSE. This link, mediated through miRNAs, establishes a mechanism for CSE-induced lung carcinogenesis.

Key Words: inflammation; epithelial-mesenchymal transition; cigarette smoke extract; carcinogenesis; miR-200c.

Cigarette smoking constitutes a major health hazard. Worldwide, approximately 1.3 billion people smoke cigarettes, which contribute to 5 million preventable deaths per year (Sridhar et al., 2008). Currently, lung cancer is the leading cause of cancer-related mortalities (Jemal et al., 2008). The most important risk factor for lung cancer is smoking (Youlden et al., 2008). Although evidence that smoking leads to a host of detrimental health outcomes, including lung cancer, is strong, the molecular mechanisms by which it causes cancer remain to be established.

Inflammation is commonly involved in development and exacerbation of lung cancer and other lung diseases (Rose-John and Schooltink, 2007). Cigarette smoke extract (CSE) induces pulmonary inflammation, which is believed to be involved in the progressive lung destruction in chronic obstructive pulmonary disease (COPD) (Barnes, 2008). Associated with COPD are increases in the levels of a cascade of inflammatory mediators such as interleukin-6 (IL-6; Barnes, 2003, 2008). IL-6 also participates in inflammation-associated carcinogenesis; the associated mechanisms involve gene modulation, an increase of invasiveness, and promotion of angiogenesis (Rose-John et al., 2007). The inflammatory conditions elicited by extrinsic environmental factors lead to malignant cell transformation, tumor growth, and metastasis (Wang et al., 2010).

The epithelial-mesenchymal transition (EMT) is a process through which cells lose their epithelial traits and acquire the attributes of mesenchymal cells (Stockinger et al., 2001). The exposure of cells to carcinogens induces EMT during transformation and tumor formation (Tellez et al., 2011; Xu et al., 2012b), suggesting that the regulation of EMT morphology and transformation are events in response to carcinogenic exposure. CSE promotes the EMT process (Liu et al., 2010). Further, the levels of vimentin and other EMT markers are increased in lungs of smokers with COPD relative to nonsmokers (Sohal et al., 2010).

MicroRNAs (miRNAs), small, noncoding RNA molecules of 21 to 23 nucleotides, are involved in the regulation of biological processes (Cheng et al., 2005). Abnormal expression levels of miRNAs are associated with a variety of human cancers (Calin and Croce, 2006). The connection between EMT
and inflammation is further strengthened by the function of inflammation-associated miRNAs in EMT and cancer metastasis (Gebeshuber et al., 2009). The miR-200 family of miRNAs (miR-141, miR-200a, miR-200b, miR-200c, and miR-429) are regulators/inhibitors of EMT and act to maintain the epithelial phenotype by targeting the expression of the E-cadherin transcriptional repressors, ZEB1 and ZEB2 (Bracken et al., 2008; Gregory et al., 2008). Expression of the miR-200 family is downregulated in many cancers (Adam et al., 2009; Dykxhoorn et al., 2009; Li et al., 2009). Thus, it is possible that altered expression of miR-200c is associated with the process of CSE-induced carcinogenesis.

Inflammation is regarded as an inducer of EMT in cancer progression, and EMT is considered to be a bridge between inflammation and cancer (Sansone and Bromberg, 2011; Zhou et al., 2012). Although molecular mechanisms for the connection between EMT and inflammation-associated miRNAs are known, it has not been determined whether EMT and inflammation-associated miRNAs contribute to CSE-induced malignant transformation and subsequent tumor formation.

As described in the present report, the roles of inflammation and EMT in the malignant transformation caused by CSE were investigated with human bronchial epithelial (HBE) cells. Chronic exposure to CSE increased IL-6 release by activating nuclear factor-κB (NF-κB), which suppressed miR-200c expression and promoted EMT and cellular transformation. Thus, a link from inflammation to EMT through miR-200c has been established. This process is apparently involved in malignant transformation and tumor formation caused by CSE. Such information contributes to an understanding of how lung cancer is caused by smoking.

MATERIALS AND METHODS

Cell culture and reagents. Simian virus 40 (SV40)-transformed HBE cells are nontumorigenic and retain features of HBE cells. They are useful for studies of multistage bronchial epithelial carcinogenesis (Reddel et al., 1988). A549 cells are human lung adenocarcinoma epithelial cells and often used as positive controls of lung cancer research. HBE and A549 cells were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). HBE and A549 cells were maintained in 5% CO₂ at 37°C in Minimum Essential Medium Eagle’s medium (MEM) or Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum (FBS, Life Technologies/Gibco, Grand Island, NY), 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies/Gibco, Gaithersburg, MD). For chronic exposure, 1 × 10⁵ cells were seeded into 10-cm (diameter) dishes for 24 h and exposed to 0 or 20 μg/ml of CSE for 24–48 h per passage. This process was continued for about 20 weeks (40 passages). Human IL-6-neutralizing antibody (IL-6 MAb, 0.5 μg/ml, Clone 6708, R&D Systems), or 0.5 μg/ml negative control anti-IgG (R&D Systems) added over the solidified agar. IL-6 was added to the medium and the cells were cultured in the presence of IL-6-neutralizing antibody, then cultures were fed every 3 days. After 14 days, colonies with > 30 cells were counted.

ELISA assays. To determine the amount of inflammatory cytokines produced by the cells, ELISAs were performed according to the manufacturer’s instructions. Cells were plated at 1 × 10⁴ per 100-cm² dish. At confluency, cell culture supernatants were harvested, centrifuged, and placed at −70°C. A human-specific IL-6 ELISA from Beijing 4A Biotech Co., Ltd (Beijing) was used to determine the amounts of inflammatory cytokines present in the supernatant. All samples were measured in duplicate, and experiments were repeated thrice. The lower limit of detection of IL-6 was 2 pg/ml.

Quantitative real-time PCR. Total cellular RNA was isolated by use of TRizol (Invitrogen) according to the manufacturer’s recommendations. For detection of mature miR-200c, 2 μg of total RNA, miRNA-specific stem-loop RT primers, and MMLV reverse transcriptase (Promega Corp., Madison, WI) were used in reverse transcription following the manufacturer’s protocol. The RT primers for miR-200c and U6 small nuclear RNA (snRNA) were as follows: miR-200c, 5′-GCCAGGTGAGGTCG-3′; U6-R, 5′-AGATATGGAAGACAAGCCGG-3′; and U6 snRNA, 5′-AAAAATATGGAACGCTTCACGAATTTGCGTGTCATCCTTGC-3′. The sequences of mature miRNAs were from Sanger miRBase (http://microrna.sanger.ac.uk/sequences/; http://www.mirbase.org/cgi-bin/mirna_entry.pl?acc=MIR000650).

Preparation of CSE. Aqueous CSE was used to mimic the effects of cigarette smoke. The cigarettes were prepared as the standard of the University of Kentucky Reference Cigarette 1R4F (9 mg tar and 0.8 mg nicotine/cigarette) (Hsu et al., 1991). The “tar” or particulate phase of smoke was collected under standard Federal Trade Commission conditions (35 ml puff volume of 2-s duration) (Narayan et al., 2004). The smoke was bubbled through serum-free MEM, and the resulting suspension was adjusted to pH 7.4 and then passed through a 0.22-μm pore filter (Schleicher & Schuell GmbH, Dassel, Germany) to remove bacteria and large particles. To prevent possible inactivation of compounds, the CSE was kept in the dark at −80°C. Before each experiment, the frozen CSE stock solution was defrosted and diluted to the desired concentrations with cell medium.

Western blots. Total cell lysates were separated by SDS-PAGE, and proteins were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA). The immune complexes were detected by enhanced chemiluminescence (Cell Signaling Technology, Beverly, MA). Antibodies used were those for p65, p-p65 (Ser 536), E-cadherin, vimentin, and N-cadherin (Cell Signaling Technology) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Sigma). Blots were quantitated by densitometry and normalized by use of GAPDH to correct for divergences in protein loading. For densitometric analyses, protein bands on the blots were measured by the use of Easy Eye II software.

Immunostaining. Immunostaining was performed as described previously (Xu et al., 2012a). Briefly, HBE cells were stained with rabbit phospho-p65 (p-p65), E-cadherin, or vimentin antibodies at 4°C overnight and then incubated with Cy3-conjugated goat-anti-rabbit secondary antibody (Millipore) for 1 h. To stain the nuclei, 4′,6-diamidino-2-phenylindole (DAPI, Sigma) was added for 10 min, and the cells were observed under a fluorescence microscope (Zeiss, LSM700B, Germany). The fluorescence intensities were measured with a multimode microscope reader (TECAN, Trading, AG, Switzerland), and images were analyzed with an Image-Pro Plus 6.0 (Olympus).

Anchorage-independent growth. Soft agar dishes were prepared with underlayers of 0.70% agarose in MEM medium supplemented with 10% FBS, as described previously. To test for capacity for soft-agar growth, HBE cells were plated in triplicate at a density of 1 × 10⁴ in 2ml of 0.35% agar in MEM medium containing human recombinant IL-6 (10 ng/ml, R&D Systems), human IL-6-neutralizing antibody (IL-6 MAb, 0.5 μg/ml, Clone 6708, R&D Systems), or 0.5 μg/ml negative control anti-IgG (R&D Systems) added over the solidified agar. IL-6 was added to the medium and the cells were cultured in the presence of IL-6-neutralizing antibody, then cultures were fed every 3 days. After 14 days, colonies with > 30 cells were counted.

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5′-GGAGTGGGGTGCAGCTGT-3′. All of the primers were synthesized by Invitrogen. Quantitative real-time PCR was performed with an Applied Biosystems 7300HT machine and MaximaTM SYBR Green/ROX qPCR Master Mix (Fermentas). U6 snRNA and GAPDH were used as internal controls to determine relative miRNA and mRNA expressions. Fold changes in expression of each gene were calculated by a comparative threshold cycle (CT) method using the formula $2^{-ΔΔCt}$ (Livak and Schmittgen, 2001). The PCR reaction was evaluated by melting curve analysis and by checking the PCR products on 2% wt/vol agarose gels.

**Statistical analysis.** Derived values are presented as the means ± SD. Comparison of mean data among multiple groups was analyzed by one-way ANOVA, and a multiple range least significant difference was used for inter-group comparisons. Values of $p < 0.05$ were considered statistically significant. All statistical analyses were performed with SPSS 16.0.

**RESULTS**

**CSE Induces EMT and Transformation of HBE Cells**

Smoking is a substantial risk factor for lung cancer, and CSE induces malignant transformation of cells and lung cancers (Shields, 1999). To investigate the effects of CSE on cell transformation, HBE cells were exposed to CSE (0, 5, 10, 20, 50, or 100 μg/ml) for 24 or 48 h. Cell viability was not greatly affected in cells incubated with CSE at concentrations of 0, 5, 10, or 20 μg/ml for 24 or 48 h; however, viability was decreased by concentrations of 50 or 100 μg/ml at 48 h. Therefore, the cells were routinely exposed to CSE at a concentration of 20 μg/ml for following experiments, the maximum concentration causing no changes in cell viability (Supplementary fig. S1).

HBE cells chronically exposed to CSE (0 or 20 μg/ml) for about 20 weeks (40 passages) were evaluated for their capacity for anchorage-independent growth, a characteristic of transformed cells (Cox and Der, 1994). In agar, 518 ± 277 colonies were formed by HBE cells exposed to CSE (20 μg/ml), and 677 ± 91 colonies were formed by A549 cells, used as a positive control. In contrast, control cells (normal HBE cells and passage-control HBE cells) showed no anchorage-independent growth (Figs. 1A and B). These results suggested that CSE induces transformation of HBE cells.

For HBE cells, the alteration from epithelial to spindle-like mesenchymal morphology was a manifestation of CSE-induced transformation, indicating that chronic exposure of CSE caused EMT (Fig. 1C). To establish that CSE induces EMT, expressions of the EMT markers, E-cadherin, vimentin, and N-cadherin were determined. After chronic exposure of cells to CSE, the expression of the epithelial marker, E-cadherin, was decreased. In contrast, expressions of the mesenchymal markers, vimentin and N-cadherin, were increased (Figs. 1D and E). To determine whether the molecular alterations of EMT occurred in control and transformed HBE cells, staining of E-cadherin and vimentin, measured by immunofluorescence microscopy, confirmed the EMT-associated shift in the localization of markers. The transformed cells formed epithelial-like intercellular junctions and displayed increased expression of fibroblast markers (Fig. 1F). Hence, both molecular and morphological changes demonstrated that, with chronic exposure to CSE, HBE cells underwent an EMT.

**Activation of p65, an Increase of IL-6 Production, and a Decrease in miR-200c Levels Are Evident During CSE-Induced Transformation of HBE Cells**

NF-κB is a transcription factor that is induced by various carcinogens and growth factors (Aggarwal and Shishodia, 2004).

To investigate the molecular alterations of EMT, the expression of the epithelial marker, E-cadherin, vimentin, and N-cadherin were determined. After chronic exposure of cells to CSE, there were increased expressions of phosphorylated p65 (p-p65) (Figs. 2A and B). As a major risk factor, chronic inflammation is common in development and exacerbation of lung cancer and in other lung diseases (Mantovani et al., 2008). Thus, in this study, we assessed the expression and secretion of IL-6. During the CSE-induced transformation of HBE cells, there were increased levels of mRNA for IL-6 (Figs. 2A and C) with increased time of exposure to CSE. Further, the secretion of IL-6 from transformed cells was examined by ELISA. The levels of IL-6 in the medium were greater for the transformed cells, relative to those for unexposed HBE cells (Fig. 2D).

Members of the miR-200 family, which includes miR-141, miR-200a, miR-200b, miR-200c, and miR-429, are regulators/inhibitors of EMT and act to maintain the epithelial phenotype (Bracken et al., 2008). To determine whether there were changes in levels of members of the miR-200 family in CSE-induced malignant transformation, the levels of miR-200a, miR-200b, miR-200c, and miR-141 in CSE-transformed cells were compared. There were lower levels of miR-200c in CSE-transformed cells (Fig. 2E). In contrast, levels of the other miR-200 family members were not significantly changed (Supplementary fig. S2).

**CSE Causes Activation of p65, Increases of IL-6 Production, and Decreases of miR-200c Levels in HBE Cells**

To determine whether CSE-induced transformation of HBE cells causes changes in p65 activation, IL-6 production, and miR-200c levels, HBE cells were exposed to CSE (0 or 20 μg/ml) for 0, 6, 12, or 24 h. There were increases in p-p65 expression with increased time of exposure to CSE (Figs. 3A and B). CSE caused an increase in IL-6 mRNA after 6 h of exposure; the increase continued progressively up to 24 h (Figs. 3A and C). Moreover, the release of IL-6 from these cells into the medium was also examined. When HBE cells were exposed to CSE for 12 or 24 h, the secretion of IL-6 was elevated (Fig. 3D). Thus, CSE enhanced the expression and the secretion of IL-6. Furthermore, to establish the effect of CSE on miR-200c expression, miR-200c levels were determined in HBE cells after CSE exposure over periods ranging from 0 to 24 h. The miR-200c levels were decreased after 6 h, confirming that the miR-200c expression was affected by CSE (Fig. 3E). These results show that, for HBE cells, CSE activates NF-κB, increases proinflammatory cytokine production, and decreases miR-200c levels.
NF-κB Is Involved in CSE-Induced Increases of IL-6 Production and Decreases of miR-200c Levels in HBE Cells

NF-κB is a transcription factor associated with the inflammatory response to cigarette smoke in the lung (Yang et al., 2007). The effects of NF-κB on production of the proinflammatory cytokine, IL-6, and on miR-200c levels in HBE cells exposed to CSE were determined. RelA/p65 subunit translocation is a key event in NF-κB activation (Baeuerle and Henkel, 1994). To investigate NF-κB activation by CSE, we tested for p65 nuclear translocation with an immunostaining assay. The results showed that, in HBE cells, p65 was strictly cytoplasmic prior to stimulation and that the localization changed to nuclear when cells were stimulated with CSE (Fig. 4D, left).
In addition, inhibition of NF-κB decreased the levels of p-p65 and the nuclear translocation of p65 induced by CSE (Figs. 4A, B, D, right, and E). Moreover, inhibition of NF-κB abolished the increases of IL-6 expression induced by CSE (Figs. 4A and C), and, in the presence of the NF-κB inhibitor, the decreased amounts of IL-6 released into the medium correlated with a decrease of IL-6 mRNA (Fig. 4C).

Because NF-κB is a regulator of miR-200c in several cell types (Chua et al., 2007), the relationship between the decreases of miR-200c and the activation of NF-κB in CSE-treated cells was assessed. Inhibition of NF-κB reversed the CSE-induced decreases of miR-200c expression (Fig. 4F). These results indicate that, in HBE cells, NF-κB is involved in the CSE-induced increase of IL-6 production and the decrease of miR-200c levels.

NF-κB, Through IL-6, Is Involved in the CSE-Induced Decreases of miR-200c Levels in HBE Cells

EMT is considered to be the convergence point between inflammation and cancer development (Sansone and Bromberg, 2011; Singh and Settleman, 2010), and IL-6 induces EMT in various cell types (Lederle et al., 2011; Yadav et al., 2011). The function of IL-6 in the CSE-induced decreases of miR-200c was validated in experiments involving use of a specific IL-6-neutralizing antibody (Hollins et al., 2008). Exposure of cells to this antibody suppressed the increase in secretion of IL-6 into the culture medium (Fig. 5A). Moreover, IL-6 neutralization led to restored expression of miR-200c (Fig. 5B). To evaluate the effects of IL-6 mediated by NF-κB on CSE-induced decreases of miR-200c expression, the role of IL-6 in miR-200c expression was examined by adding recombinant IL-6 protein to the
NF-κB-inhibited cells. The increase of miR-200c expression by inhibition of NF-κB was reversed (Fig. 5C). Thus, in HBE cells, NF-κB, acting via IL-6, was involved in the CSE-induced decreases of miR-200c levels.

**Effects of IL-6 on Maintenance of CSE-Induced Transformation and on Malignant Progression of HBE Cells**

IL-6, which affects the malignant behavior of cells, enhances cell transformation induced by benzopyrene diol epoxide in Beas-2B cells (Chen et al., 2012). The function of IL-6 in CSE-induced transformation, an early step in lung carcinogenesis, was examined. The capacity of colony formation by HBE cells exposed to CSE for 40 passages with or without IL-6 was determined by use of IL-6-neutralizing antibody (Hollins et al., 2008). These cells, depleted of IL-6 by the antibody, displayed fewer colonies compared with the untreated group (Figs. 6A and B). Thus, IL-6 neutralization decreased the neoplastic capacity of these cells, showing that IL-6 was involved in their CSE-induced malignant transformation.

**NF-κB Is Involved in CSE-Induced EMT and Transformation of HBE Cells Through Production of IL-6**

Because NF-κB is involved in the CSE-induced increases of IL-6 production and because IL-6 is necessary for maintenance of CSE-induced transformation and for malignant progression of HBE cells, the role of NF-κB, acting via IL-6, in neoplastic transformation of HBE cells induced by CSE was evaluated. HBE cells were exposed to CSE (0 or 20 μg/ml) in the absence or presence of Bay11-7082 for about 20 weeks (40 passages). After 20 weeks, anchorage-independent growth of treated cells was evaluated. In agar, 452 ± 96 colonies were formed from HBE cells exposed to CSE. In contrast, control cells and cells exposed to Bay11-7082 with or without CSE showed no anchorage-independent growth (Figs. 7A and B). In cells chronically exposed to CSE and treated with Bay11-7082, there were no changes in levels of p-p65, E-cadherin, vimentin, and N-cadherin relative to untreated controls (Figs. 7C, D, and E). Inhibition of NF-κB blocked the CSE-induced increases of IL-6 expression and secretion (Figs. 7F and G). There were decreases of miR-200c levels in CSE-transformed cells;
INFLAMMATION TO EMT IN CSE CARCINOGENESIS

However, in cells exposed to CSE in the presence of Bay11-7082, decreases of miR-200c levels were not evident (Fig. 7H). Hence, NF-κB, acting through IL-6, was involved in the CSE-induced EMT and transformation of HBE cells.

**DISCUSSION**

Tobacco smoke is a complex chemical mixture containing over 4,000 different compounds, more than 50 of which are known carcinogens, cocarcinogens, and/or mutagens (Chen et al., 2005; Hoffmann and Hoffmann, 1997). Epidemiological evidence confirms that exposure to cigarette smoke increases the incidence of lung carcinogenesis, a leading cause of cancer deaths in the United States and other developed countries (Sasco et al., 2004). Characterization of the mechanisms by which chemical carcinogens transform human cells will lead to a greater understanding of the molecular events that program the malignant state.

To investigate the mechanisms of lung tumorigenesis induced by tobacco smoke, we used CSE to mimic the effects of cigarette smoke. The viability of HBE cells exposed to CSE at concentrations of 0, 5, 10, 20, 50, or 100 μg/ml was assessed. Although there were slight, but significant, increases in viability (p < 0.05) for concentrations of 10, 20, and 50 μg/ml at 24 h and for concentrations of 10 and 20 μg/ml at 48 h, these differences may not be biologically significant. In contrast, there were substantial decreases in the viability of cells exposed to CSE at a concentration of 100 μg/ml for 24 h and at concentrations of 50 and 100 μg/ml for 48 h. Thus, CSE at a concentration of 20 μg/ml was selected for repeated, long-term exposure of cells (24–48 h per passage). This level of CSE resulted in neoplastic transformation of HBE cells, as determined by anchorage-independent growth in soft agar.

EMT, which occurs during normal embryonic development, features a loss of epithelial properties and acquisition of mesenchymal properties (Nakamura and Tokura, 2011). The EMT is viewed as a step in tumor invasion and metastasis (Thiery and Sleeman, 2006). Exposure of cells to arsenite or other carcinogens induces EMT during transformation and tumor formation (Tellez et al., 2011; Wang et al., 2011), suggesting that EMT is involved in cell transformation induced by carcinogen exposure. As described in the present effort, chronic CSE exposure induced the EMT in HBE cells. Thus, CSE-induced EMT was associated with transformation.

Members of the miR-200 family (miR-141, miR-200a, miR-200b, miR-200c, and miR-429) are regulators/inhibitors of the EMT and act to maintain the epithelial phenotype.
Further, in cancers, expression of miR-200 family members is generally downregulated (Bracken et al., 2008; Li et al., 2009). Our results show that the expression of miR-200c is decreased in HBE cells after acute or chronic exposure to CSE; the levels of three other miR-200 family members are not significantly changed. These results suggest that miR-200c is involved in CSE-induced EMT.

Exposure of humans to cigarette smoke leads to pulmonary inflammation, which is believed to be involved in the progressive lung destruction in COPD (Barnes, 2008). CSE induces the proinflammatory COX-2 protein in human lung fibroblasts (Martey et al., 2004) and increases production of the inflammatory cytokines, IL-6 and IL-8, in IL-1β-activated HMC-1 cells (Chi et al., 2012). There have been numerous attempts to characterize the molecular and cellular mechanisms for the effects of CSE on inflammation that is related to health effects.

IL-6 is a chemotactic cytokine that, in the human lung, is involved in recruiting neutrophils and macrophages to sites of inflammation (Grivennikov and Karin, 2011); it is an inflammatory mediator associated with COPD (Barnes, 2003, 2008). The results reported here show that the expression and secretion of IL-6 are induced by CSE in HBE cells within 24 h and during their CSE-induced transformation; these observations are in agreement with other data (Chi et al., 2012). Based on these results, we believe that CSE induces inflammation in HBE cells.

The expression of various inflammatory cytokines is regulated by transcription factors (Rahman and Adcock, 2006; Rajendrasozhan et al., 2009). Some reports show that NF-κB is involved in inflammation and COPD induced by cigarette smoke (Yang et al., 2006). Site-specific posttranslational modifications, such as phosphorylation of RelA/p65 (a subunit of NF-κB), are necessary for NF-κB activation and for CSE-mediated lung inflammation (Chen et al., 2005; Yang et al., 2007). We found that CSE induces activation of NF-κB in HBE cells. Activation of NF-κB is involved in inflammation through its capacity to induce the transcription of proinflammatory genes (Rodriguez-Roisin and Soriano, 2008). Because, in HBE cells, NF-κB regulates secretion of the proinflammatory chemokine, IL-6, the present results are in agreement with other reports that activation of NF-κB is necessary for transcription of IL-6 (Rahman, 2003). Together, these data suggest that NF-κB regulates the inflammation induced by CSE in HBE cells.

miR-200c has been implicated in the process of EMT (Cano and Nieto, 2008; Korpal and Kang, 2008). Moreover, NF-κB has been identified as a regulator of the EMT in several cell types (Chua et al., 2007). As demonstrated in this study, CSE induced the activation of NF-κB and downregulated the expression of miR-200c. Accordingly, we further hypothesized that the activation of NF-κB is associated with the decrease of miR-200c in HBE cells exposed to CSE. Our results showed that inhibition of NF-κB activation reversed the CSE-induced decrease of miR-200c expression. These data indicated that NF-κB regulated the decrease of miR-200c in these cells.
Regarding NF-κB, various reports show that NF-κB inhibitors such as Bay 11-7082 and IKK inhibitors can affect inflammation independent of their inhibitory effect on the NF-κB pathway (Bauerle et al., 2010). In order to eliminate the possible inhibition of inflammation by Bay 11-7082 independent of NF-κB, we inhibited NF-κB activation by p65 siRNA to test the effects of NF-κB on the expression and secretion of IL-6 and miR-200c expression. The results showed that knockdown of p65 by siRNA abolished the increases of IL-6 expression and decreases of miR-200c expression, results that are consistent with the application of Bay11-7082. Based on these results, we conclude that Bay11-7082 inhibits IL-6 secretion through its effects on the NF-κB pathway, not by inhibiting the inflammatory response through another mechanism.

EMT has been implicated in chronic inflammation, fibrosis, and cancer development (Gotzmann et al., 2004; Kalluri and Neilson, 2003). The connection between inflammation and EMT progression in development of lung cancer and resistance to therapy has been emphasized (Dohadwala et al., 2006; Krysan et al., 2008). Furthermore, the connection between EMT and inflammation is strengthened by studies on the role of inflammation-associated miRNAs in EMT and metastasis (Gebeshuber et al., 2009). We hypothesized that the expression of miR-200c decreased by CSE was dependent on CSE-induced production of IL-6. Accordingly, neutralization of IL-6 led to recovery of miR-200c expression after exposure to CSE, indicating that the downregulation of miR-200c levels in cells was a result of increased IL-6 after CSE exposure. In addition, NF-κB regulated the production of IL-6 in HBE cells exposed to CSE. To elucidate the effects of IL-6 mediated by NF-κB on CSE-induced EMT, expression of miR-200c was examined in the presence of recombinant IL-6 protein in cells with inhibited NF-κB. Data showed that IL-6, dependent on NF-κB activation, was involved in CSE-regulated miR-200c expression. Based on these data and on those previously reported, we concluded that chronic exposure to CSE increased IL-6 release by activating NF-κB, which suppressed miR-200c expression.

The secretion of IL-6 most likely involves an autocrine signaling loop that induces signaling through cell surface receptors and activation of downstream signaling pathways, such as the JAK/Stat pathway (Teng et al., 2013). We blocked the activation of the JAK/Stat pathway by a STAT3 inhibitor to determine the effects of inhibition of IL-6 signaling on the expression of miR-200c. We found that inhibition of IL-6 signaling in this manner reversed the CSE-induced decreases of miR-200c. These results strengthen our conclusion that chronic exposure of cells to CSE increases IL-6 release by activating NF-κB, which suppresses miR-200c expression.

Chronic inflammation is linked with malignant transformation, tumor growth, and, possibly, tumor metastasis (Karin et al., 2006; Takeuchi and Akira, 2010). In Beas-2B cells, IL-6, which affects the malignant behavior of cells, enhances the cell transformation induced by benzopyrene diol epoxide (Chen et al., 2012). Investigations of the role of IL-6 in CSE-induced transformation and malignant behavior show that HBE cells with downregulated IL-6 and exposed to CSE for 40 passages develop fewer colonies, an observation consistent with the report that IL-6 is involved in cell transformation induced by other environmental chemicals (Chen et al., 2012).

Considering literature reports and our data, we suggest that NF-κB activation is a link between chronic inflammation, EMT, and lung cancer induced by CSE. Our data show that inhibition of NF-κB activation blocks CSE-induced malignant transformation of HBE cells, reduces the expression of E-cadherin and miR-200c, and elevates IL-6 and N-cadherin. Together, the results indicate that, in HBE cells, NF-κB is involved in the neoplastic transformation induced by CSE through CSE-induced inflammation and EMT. In this study, we established that the miR-200 family is involved in the CSE-induced malignant transformation of cells although we did not perform tests of other miRNAs. We recognize that other miRNAs, such as miR-638, are involved in the malignant transformation of cells (Li et al., 2012). Accordingly, in further studies, we intend to assess more miRNAs, which may be related to human lung cancer induced by CSE or by a single chemical such as NNK or BaP.

In summary, we have provided evidence that chronic exposure of cells to CSE induces an inflammatory response that contributes to CSE-induced EMT and malignant transformation. We have shown that the CSE-induced downregulation of miR-200c depends on NF-κB activation through production of

**FIG. 6.** Effects of IL-6 on maintenance of CSE-induced transformation and malignant progression of HBE cells. C-HBE, passage-control HBE cells; T-HBE, CSE-transformed HBE cells. HBE and T-HBE cells were cultured in the presence of anti-IL-6 antibody or negative control anti-IgG for 3 days and then seeded in soft agar with medium containing anti-IL-6 antibody (0.5 μg/ml) over the agar. (A) Cell colonies and (B) their numbers (means ± SD, n = 3) in soft agar for HBE and T-HBE cells. Bars = 100 μm. *p < 0.05 difference from T-HBE cells in the absence of anti-IL-6 antibody.
These results provide a relevant link, through miRNAs, between the inflammation and EMT, which contributes a possible mechanism for CSE-induced carcinogenesis.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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