

Appalachian Mountaintop Mining Particulate Matter Induces Neoplastic Transformation of Human Bronchial Epithelial Cells and Promotes Tumor Formation

Sudjit Luanpitpong,^{*,†,‡,§} Michael Chen,^{†,‡} Travis Knuckles,[¶] Sijin Wen,^{||} Juhua Luo,[▲] Emily Ellis,[#] Michael Hendryx,[▲] and Yon Rojanasakul^{†,‡}

[†]Department of Pharmaceutical Sciences, West Virginia University, Morgantown, West Virginia 26506, United States

[‡]Mary Babb Randolph Cancer Center, West Virginia University, Morgantown, West Virginia 26506, United States

[§]Siriraj Center of Excellence for Stem Cell Research, Mahidol University, Bangkok 10700, Thailand

[¶]Center for Cardiovascular and Respiratory Science, West Virginia University, Morgantown, West Virginia 26506, United States

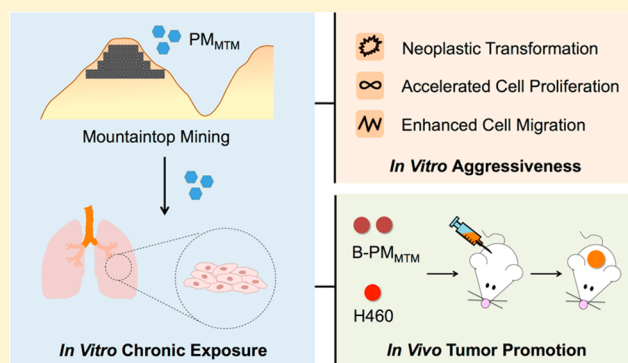
^{||}Department of Biostatistics, West Virginia University, Morgantown, West Virginia 26506, United States

[▲]School of Public Health, Indiana University, Bloomington, Indiana 47405, United States

[#]Animal Models and Imaging Facility, West Virginia University, Morgantown, West Virginia 26506, United States

S Supporting Information

ABSTRACT: Epidemiological studies suggest that living near mountaintop coal mining (MTM) activities is one of the contributing factors for high lung cancer incidence. The purpose of this study was to investigate the long-term carcinogenic potential of MTM particulate matter (PM_{MTM}) exposure on human bronchial epithelial cells. Our results show that chronic exposure (3 months) to noncytotoxic, physiological relevant concentration (1 μg/mL) of PM_{MTM}, but not control particle PM_{CON}, induced neoplastic transformation, accelerated cell proliferation, and enhanced cell migration of the exposed lung cells. Xenograft transplantation of the PM_{MTM}-exposed cells in mice caused no apparent tumor formation, but promoted tumor growth of human lung carcinoma H460 cells, suggesting the tumor-promoting effect of PM_{MTM}. Chronic exposure to the main inorganic chemical constituent of PM_{MTM}, molybdenum but not silica, similarly induced cell transformation and tumor promotion, suggesting the contribution of molybdenum, at least in part, in the PM_{MTM} effects. These results provide new evidence for the carcinogenic potential of PM_{MTM} and support further risk assessment and implementation of exposure control for PM_{MTM}.



INTRODUCTION

Lung cancer is the leading cause of cancer-related death, and, after smoking, environmental and occupational exposure is a major cause.^{1,2} The Appalachian Mountains stretch across 13 states of the United States from southern New York to northern Mississippi. Health disparities, most notably cancer incidence and mortality rate, are higher in the Appalachian region compared to the rest of the country.^{3,4} Previous epidemiology studies demonstrated elevated lung cancer mortality in coal-mining areas of Appalachia,^{5,6} suggesting that environmental contaminants from coal-mining activities may contribute to the increased lung cancer risk.

Mountaintop removal mining (MTM) is a major form of surface coal mining in Appalachia, especially in West Virginia and Kentucky.⁷ In southern West Virginia, almost 40 million tons of coals were extracted by MTM in 2012.⁸ Particulate matter (PM) is generated from these active MTM sites by blasting and combustion from heavy equipment and may represent a

potential toxicant that is elevated in ambient air.⁹ The lungs are the primary target organ for these airborne MTM-derived PM (PM_{MTM}) exposures.¹⁰ To date, there have been no experimental reports on the potential carcinogenic effect of PM_{MTM}, either in vitro or in vivo. Because carcinogenesis is a multistep process commonly associated with long-term exposure to carcinogens,^{11,12} we studied the chronic effects of PM_{MTM} exposure on human bronchial epithelial cells, one of the major cellular targets of lung carcinogenesis. Such information is necessary to provide a scientific basis for the epidemiological finding on increased lung cancer mortality in the coal-mining areas of Appalachia.

Received: June 11, 2014

Revised: October 12, 2014

Accepted: October 14, 2014

Published: October 14, 2014

In the present study, we chronically exposed human bronchial epithelial BEAS-2B cells to noncytotoxic, physiologically relevant concentration of PM_{MTM} or control PM (PM_{CON}) over a 3-month period in culture. The exposed lung cells were then evaluated for their neoplastic transformation, proliferative, and migratory properties in vitro and tumorigenicity in vivo. We also studied the effect of inorganic chemical constituents of PM_{MTM} by similarly exposing bronchial epithelial cells to silica (Si) and molybdenum (Mo), the main inorganic chemical constituents of PM_{MTM}. Our data indicate the cell-transforming and tumor-promoting effects of PM_{MTM}; thus supporting the prudent adoption of prevention strategies and implementation of exposure control for PM_{MTM}. The described chronic exposure model could further be used for mechanistic studies and risk assessment of PM_{MTM}, which may not be feasible in vivo.

MATERIALS AND METHODS

A more detailed description of Materials and Methods used in this study is available as Supporting Information at <http://pubs.acs.org/>.

Cell Culture. Human bronchial epithelial BEAS-2B and nonsmall cell lung cancer H460 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA) and were cultured as described previously.¹³

Collection of MTM and Control Particulate Matters. Air samples were taken at two rural residential sites located within 1 mile of an active MTM site in Edwight, WV, U.S.A. For control, air was similarly sampled from selected rural areas in Green Bank, WV, which does not have coal mining.¹⁴ PM_{MTM} and PM_{CON} were collected on PTFE fiber-backed filters with a pore size of 5 μm (Whatman, Springfield Mill, U.K.) for 2–4 weeks. The filters were extracted according to the method previously described (see Supporting Information Table S1 for PM mass).¹⁵ It is worth noting that this method of PM collection could not preserve the volatile organic compounds. Scanning electron microscope-energy-dispersive X-ray spectroscopy (SEM-EDX), which was limited to the analysis of inorganic compounds, was further used to perform PM compositional analysis (RTI International, Research Triangle Park, NC). In comparison with PM_{CON}, Si and Mo were found to be the main inorganic chemical elements in PM_{MTM} with the % weight-average of $48.15 \pm 26.91\%$ and $28.90 \pm 4.16\%$, respectively, for Si and Mo versus $23.75 \pm 15.07\%$ and $0.00 \pm 0.00\%$ of the elements in PM_{CON} (see Table S2 for analysis of organic elements).

Cytotoxicity Assay. Cell viability was determined by MTT assay, as described previously.¹⁶ All particles were suspended in phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA) and were lightly sonicated prior to use to disperse the particles. The absorbance ratio of MTT formazan product of treated and nontreated cells was calculated and presented as relative cell viability.

Chronic Particle Exposure. Subconfluent cultures of bronchial epithelial BEAS-2B cells were continuously exposed to noncytotoxic concentration (1 $\mu\text{g}/\text{mL}$) of PM_{MTM} or PM_{CON} in 6-well plates for 3 months and were passaged biweekly. PM_{MTM}- and PM_{CON}-exposed BEAS-2B cells were designated as B-PM_{MTM} and B-PM_{CON} cells. Parallel culture grown with the same background level of dispersant provided a passage-matched control (B-NTX cells). To study the effect of PM_{MTM} inorganic chemical elements, cells were similarly exposed to a noncytotoxic concentration (1 $\mu\text{g}/\text{mL}$) of Si or Mo for 3 months (designated as B-Si and B-Mo cells). All cells were cultured in complete

medium (without treatment) for at least 10 passages prior to experiments to rule out any reversible effects.

Dosage Calculation and Human Extrapolation. PM exposure dose of 1 $\mu\text{g}/\text{mL}$ in the 6-well plates (growth area $\sim 10\text{ cm}^2$) at the total volume of 1 mL corresponds to the surface area dose of 0.1 $\mu\text{g}/\text{cm}^2$. Based on the reported rat lung surface area of 5000 cm^2 ,¹⁷ this exposure dose is equivalent to a bolus exposure of PM at 0.5 mg in the rats, which was previously shown to induce pathological changes.¹⁸ Assuming the pulmonary surface area in humans of 100 m^2 , the human burden is equal to 100 mg/lung. Considering a respiratory deposition of $\sim 40\%$ ¹⁴ and an adult inhalation rate of $\sim 16\text{ m}^3/\text{day}$,¹⁹ the experimental dose could be reached within 8.5 years of human inhalation exposure at 5 $\mu\text{g}/\text{m}^3$ (average total PM mass concentration in Edwight and Green Bank, WV).¹⁴

Soft Agar Colony Formation Assay. The chronically exposed cells at 3×10^4 cells per 24-well plate were mixed with culture medium containing 0.5% agar. The resulting cell suspensions were immediately plated onto dishes coated with 0.5% agar in culture medium. After 2 weeks, colonies larger than 50 μm in diameter were scored as positive for growth.²⁰

Cell Counting. The exposed cells (3×10^4 cells) were seeded in 24-well plates and cultured in complete medium. The cells were stained with 0.4% trypan blue (Invitrogen) (to indicate dead cells), and healthy cell number was scored using a Countess automated cell counter (Invitrogen) at 2 and 5 days.

Proliferative Index. The chronically exposed cells (2×10^6 cells) were labeled with CellVue Claret Far Red Fluorescent Cell Linker (Sigma) according to the manufacturer's protocol. After 4 days of culture, proliferative index was determined on the basis of far red fluorescence intensity using FSC Express 4 Flow Cytometry software (De Novo Software, Los Angeles, CA).

Cell Cycle Analysis. The chronically exposed cells were serum starved for 12 h and incubated in the complete medium for 8 h. The cells were then stained with 20 $\mu\text{g}/\text{mL}$ PI and the percentage of cells in different phases of cell cycle was determined by FSC Express 4 software.

Migration Assay. Cell migration was determined by wound healing assay as previously described.¹⁶ Briefly, a monolayer of chronic exposed cells was cultured in 24-well plate, and a wound space was created with a 1 mm width tip. The cell monolayers were incubated in complete medium and allowed to migrate for 24 h.

Xenograft Mouse Model. Animal care and experimental procedure described in this study were in accordance with the Guidelines for Animal Experiments at West Virginia University (IACUC no. 12-0502). Immunodeficient NOD/SCID gamma mice, strain NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG; Jackson Laboratory, Bar Harbor, ME), were maintained under pathogen-free conditions within the institutional animal facility.^{21,22} Mice were injected subcutaneously (SC) with 3×10^5 luciferase (Luc2; Capital Biosciences, Rockville, MD)-labeled lung cancer H460 cells and 6×10^5 PM-exposed cells (1:2 ratio) suspended in 100 μL of ExtraCel hydrogel (Advanced BioMatrix, San Diego, CA). Tumor growth of luciferase-labeled cells was monitored weekly using IVIS bioimaging (PerkinElmer, Waltham, MA). At the end of experiments, mice were euthanized, and SC tumors were dissected and weighted. Metastasis of tumor cells to neighbor tissues was analyzed by IVIS imaging after removal of primary SC tumors.

Statistical Analysis. The data represent means \pm SD from three or more independent experiments as indicated. Statistical

analysis was performed by Student's *t* test at a significance level of $P < 0.05$.

RESULTS

Effects of PM_{MTM} on Cytotoxicity. The purpose of this study was to establish an experimental human lung cell model for

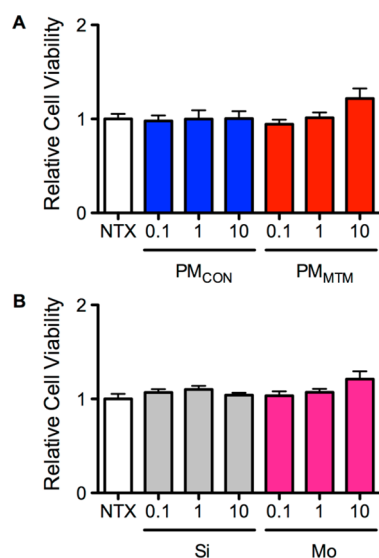


Figure 1. Effect of acute PM_{MTM} exposure on cytotoxicity of human bronchial epithelial cells. (A) Subconfluent monolayers of BEAS-2B cells were left untreated (nontreatment, NTX) or treated with various concentrations (0.1–10 $\mu\text{g/mL}$) of PM_{CON} and PM_{MTM} for 48 h and analyzed for cell viability using MTT assay. (B) Cells were treated with Si or Mo at the same concentration range and analyzed for cell viability after 48 h by MTT assay. Data are mean \pm SD ($n = 4$).

PM_{MTM} carcinogenesis studies that would allow further molecular and cellular mechanistic studies underlying cancer-like phenotypes. We first characterized the acute cytotoxic effect of PMs to determine their noncytotoxic concentrations for subsequent long-term exposure studies. Human bronchial epithelial cells were exposed to various concentrations (0.1–10 $\mu\text{g/mL}$) of PM_{CON} and PM_{MTM} for 48 h, and cell viability was determined by MTT assay. The results showed that none of the PM_{CON} and PM_{MTM} treatments caused a significant effect on cell viability as compared to nontreated (NTX) control (Figure 1A). We similarly tested the dose effect of inorganic chemical elements of PM_{MTM} (Si and Mo) on cell viability. The results similarly showed the noncytotoxic effect of Si and Mo at the treatment doses of 0.1–10 $\mu\text{g/mL}$ (Figure 1B). As we observed a slight increase in cell viability (proliferation), albeit not significant, at the high dose of PM_{MTM} (10 $\mu\text{g/mL}$), we used a lower dose (1 $\mu\text{g/mL}$) in our subsequent chronic exposure studies.

Chronic PM_{MTM} Exposure Induces Neoplastic Transformation. To mimic the long-term carcinogenic process, cells were chronically exposed to a noncytotoxic concentration of PMs at 1 $\mu\text{g/mL}$ (0.1 $\mu\text{g/cm}^2$ surface area dose) and passaged biweekly. This surface area dose mimics the in vivo dose in rodents of 0.5 mg or approximately 8.5 years of human inhalation exposure as described in Materials and Methods. In this study, the cells were exposed to PM_{MTM}, PM_{CON}, or left untreated for 3 months (Figure 2A), after which they were grown in complete medium (without treatment) for at least 10 passages and examined for anchorage-independent growth by soft-agar colony

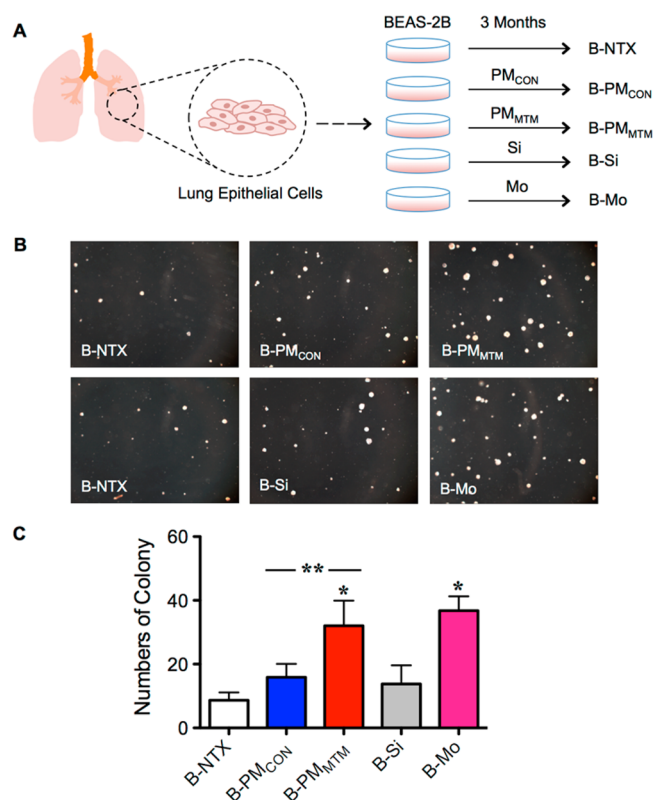


Figure 2. Chronic exposure to PM_{MTM} induces neoplastic transformation of human bronchial epithelial cells. (A) Schematic representation of chronic exposure model. BEAS-2B cells were continuously exposed to noncytotoxic concentration (1 $\mu\text{g/mL}$) of PM_{CON}, PM_{MTM}, Si, and Mo for 3 months and designated as B-PM_{CON}, B-PM_{MTM}, B-Si, and B-Mo cells, respectively. BEAS-2B cells maintained in culture without particle exposure (B-NTX) served as passage control cells. (B, C) Cells were seeded on 0.5% agar plates, and after 2 weeks they were visualized under a phase contrast microscope. (C) Quantification of large colonies ($>50 \mu\text{m}$ in diameter). Data are mean \pm SD ($n = 4$). * $P < 0.05$ (power $>95\%$) vs passage control B-NTX cells. ** $P < 0.05$ (power $>80\%$) vs B-PM_{CON} cells.

formation assay, which is one of the most stringent indicators of neoplastic transformation.²³ To determine the key inorganic constituents of PM_{MTM} that may contribute to its pathological effect, cells were similarly exposed to Si and Mo, and analyzed for cell transformation. Figure 2B,C show that as compared to particle-control B-PM_{CON} cells, the B-PM_{MTM} and B-Mo cells formed larger and greater numbers of colony, whereas the B-Si cells exhibited a similar colony forming activity. These results indicate the neoplastic transformation of B-PM_{MTM} and B-Mo cells.

Chronic PM_{MTM} Exposure Alters Cell Growth Characteristic. Excessive cell growth is one of the carcinogenic properties of malignant cells.^{24,25} To determine whether chronic PM exposure affects cell growth characteristic, the exposed cells were analyzed for cell proliferation by direct cell counting and dye-based assays. Figure 3A shows that the B-PM_{MTM} and B-Mo cells exhibited a significantly higher proliferation rate than the B-PM_{CON} cells, which grew at a similar rate as the B-NTX and B-Si cells. To confirm this result which was based on direct cell counting assay, the cells were stained with membrane dye CellVue Claret, and their proliferative index was determined by flow cytometry. This dye-based assay measures cell proliferation based on the principle of dye dilution upon cell division.

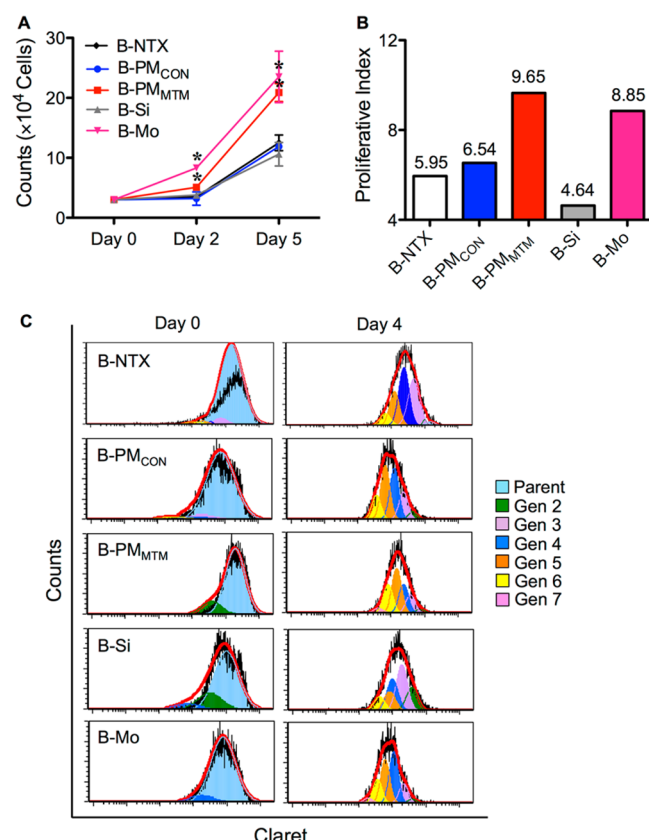


Figure 3. Chronic PM_{MTM} exposure accelerates proliferation of human bronchial epithelial cells. (A) B-PM_{CON}, B-PM_{MTM}, B-Si, B-Mo, and B-NTX cells were plated in 24-well plates at the density of 3×10^4 cells in growth medium. After 2 and 5 days, the cells were counted using an automated cell counter. Data are mean \pm SD ($n = 3$). * $P < 0.05$ (power $> 95\%$) vs passage control B-NTX cells. (B) Cells were labeled with membrane dye CellVue Claret. After 4 days of culture, cellular fluorescence intensity was determined by flow cytometry, and proliferative index was calculated. (C) Representative flow cytometric histograms from three independent experiments showing brightly stained parental cells and weakly stained daughter cells.

Consistent with the direct cell counting result, the dye-based assay indicated a higher proliferative index of B-PM_{MTM} and B-Mo cells compared to B-PM_{CON} and B-Si cells (Figure 3B). Analysis of cellular fluorescence intensity further indicated the division of parental cells with the observed seventh generation of daughter cells only in the B-PM_{MTM} and B-Mo cells (Figure 3C), thus substantiating the above finding.

To delineate the mechanism of PM_{MTM}-induced cell proliferation, we investigated the cell cycle progression of synchronized B-PM_{MTM}, B-PM_{CON}, B-Si, and B-Mo cells by flow cytometry using propidium iodide (PI) DNA staining assay. As depicted in Figure 4A, a higher percentage of B-PM_{MTM} cells compared to B-PM_{CON} cells entered the S phase ($\sim 80\%$ vs 50%) and reached the G2/M transition phase ($\sim 5\%$ vs 1%), whereas the B-Mo cells had a significant portion in the G2/M phase ($\sim 10\%$). These results indicated the promotion of S phase entry by chronic PM_{MTM} exposure and the transition to G2/M phase by chronic Mo exposure.

Chronic PM_{MTM} Exposure Promotes Cell Migration. The aggressive behavior of PM_{MTM}-exposed cells was examined by assessing their migratory activity, which is a key determinant of tumor invasion and progression.^{26,27} Cell migration was determined by scratch or wound healing assay. At 24 h after

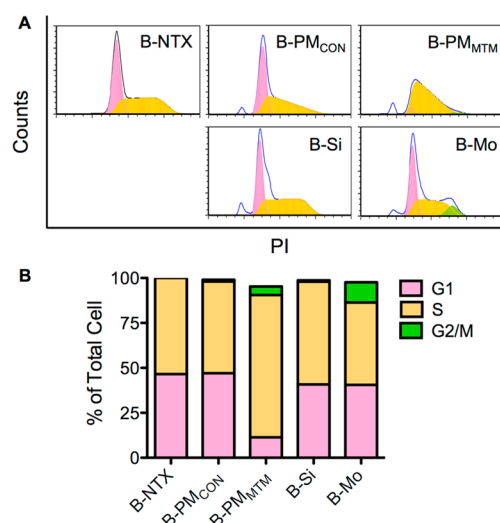


Figure 4. Chronic PM_{MTM} exposure alters cell cycle of human bronchial epithelial cells. (A) B-NTX, B-PM_{CON}, B-PM_{MTM}, B-Si, and B-Mo cells were serum starved for 12 h to synchronize their cell cycle. They were then cultured in a complete medium for 8 h and analyzed for their cell cycle by flow cytometry. Representative histograms from three independent experiments were shown. (B) Plots are percentages of total cells (50 000 events) in each phase of the cell cycle (G1, S, and G2/M).

the scratch, B-PM_{MTM} and B-Mo cells showed a significantly higher motility rate toward the wound compared to B-NTX, B-PM_{CON} and B-Si cells, as judged by their greater wound closure (Figure 5A,B). These results indicate the induction of aggressive cell behavior by chronic exposure to PM_{MTM} and Mo.

Tumorigenicity Assessment of PM_{MTM} Cells in Mice.

Carcinogenesis is a multistep sequential process consisting of three major stages, namely, initiation, promotion, and progression.^{12,28,29} Certain carcinogens can act in one or all of these stages, which results in neoplastic transformation and tumor development.³⁰ Having demonstrated the neoplastic transformation of B-PM_{MTM} cells, we next assessed their tumorigenic potential in vivo. The B-PM_{MTM} cells and their control B-PM_{CON} cells as well as B-NTX, B-Mo, and B-Si cells (1×10^6) were injected into NSG mice subcutaneously and tumor formation was determined over time. No tumor formation was observed with any of the above treatments including those injected with the neoplastic B-PM_{MTM} and B-Mo cells (Figure 6A), indicating their inherent nontumorigenicity. To test whether these cells might possess tumor-promoting activity, we coinjected the B-PM_{MTM}, B-PM_{CON}, B-Si, or B-Mo cells (6×10^5) with tumorigenic human lung cancer H460 cells (3×10^5), which have been modified to express luciferase to aid quantitation of tumor formation in mice by bioluminescence imaging (Figure 6B). Tumor luminescence signals were quantified over time and normalized to their initial signal at the time of inoculation (day 1). At 1 week postinjection, tumor luminescence was higher in mice bearing the H460 cells with B-PM_{MTM}, B-Mo, or B-Si cells as compared to the mice bearing the H460 with B-PM_{CON} cells (Figure 6C). At 2 weeks postinjection, the tumor luminescence intensity was high only in the mice injected with H460 cells and B-PM_{MTM} or B-Mo cells, but not B-PM_{CON} or B-Si cells (Figure 6D). These results indicate the tumor-promoting activity of B-PM_{MTM} and B-Mo cells.

At the end of the experiments (week 3), SC tumors were dissected and their bioluminescence were determined and

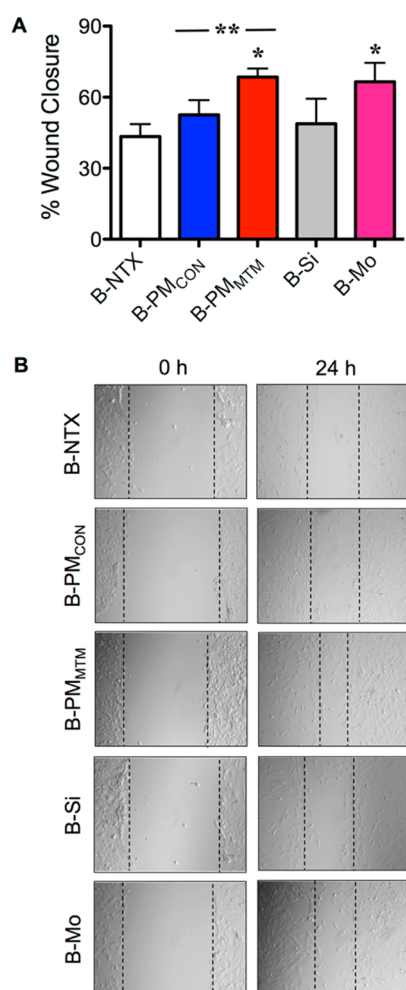


Figure 5. Chronic PM_{MTM} exposure enhances migration of human bronchial epithelial cells. (A) Confluent monolayers of B-NTX, B-PM_{CON}, B-PM_{MTM}, B-Si, and B-Mo cells were wounded, and the cells were allowed to migrate for 24 h. Wound space was visualized under a phase contrast microscope and analyzed by comparing the change in wound space as a percentage of wound closure. Data are mean \pm SD ($n = 3$). * $P < 0.05$ (power $>80\%$) vs passage control B-NTX cells. ** $P < 0.05$ (power $>80\%$) vs B-PM_{CON} cells. (B) Representative micrographs from three independent experiments were shown.

compared between groups (Figure 7A). Figure 7B shows a stronger bioluminescence signal in tumors from H460 and B-PM_{MTM} or H460 and B-Mo cells, compared to those from H460 and B-PM_{CON} or H460 and B-Si cells. Analysis of tumor weight of the samples further supported the tumor-promoting role of B-PM_{MTM} and B-Mo cells (Figure 7C). Interestingly, we observed notable tumor bioluminescence signals in mice bearing H460 and B-PM_{MTM}, B-Si or B-Mo cells after the dissection of SC tumors (Figure 7A,D), suggesting metastasis of tumor cells to neighboring tissues and strengthening the important role of chronic PM_{MTM} and Mo exposure in tumor promotion.

DISCUSSION

A growing body of evidence links living in proximity to MTM activities to greater risk of serious health consequences, including significantly higher reports of cancer.³¹ The MTM operation uses explosive and excavation equipment to remove vegetation, rock, and dirt from mountaintops to expose coal seams, and thus, it consists of active areas of blasting, crushing, and grinding.⁷

MTM activities result in the production of atmospheric PM (PM_{MTM}) that might be associated with human health effects. Currently, the direct relationship between chronic pulmonary exposure to PM_{MTM} and lung cancer risk has not been investigated. In this study, we reported a combined in vitro–in vivo model for PM_{MTM} lung carcinogenesis studies using chronically exposed human bronchial epithelial BEAS-2B cells and a mouse xenograft model. Bronchial epithelial cells were chosen in this study as they are one of the key targets for lung carcinogenesis. Airway epithelium lines the body's first physiological barrier to inhaled PM and the particles in range of 1–5 μm generally deposited in the tracheobronchial region of the airways—such deposition appears to be a close correlation with the incidence of primary cancer sites.³²

Anchorage-independent growth has been well correlated with the tumorigenicity and invasiveness of several cancer cell types.²⁴ Colony formation under soft agar assay, the gold standard test to evaluate the ability of cells to undergo anchorage-independent growth, is therefore the most stringent indicator for neoplastic transformation. We showed that chronic PM_{MTM}-exposed B-PM_{MTM} cells induced larger number and size of colonies as compared to chronic PM_{CON}-exposed B-PM_{CON} cells and passage-matched control B-NTX cells (Figure 2). It has previously been reported that BEAS-2B cells might undergo squamous differentiation in the presence of serum.^{33,34} However, because B-PM_{CON} and B-NTX cells showed no phenotypic changes or neoplastic behavior under the culture condition, it can be concluded that the B-PM_{MTM} cells, not B-PM_{CON} and B-NTX cells, have undergone differentiation and show altered phenotype due to continued exposure. In order to delineate the chemical effects of PM_{MTM} inorganic elements, bronchial epithelial cells were similarly exposed to Si and Mo and neoplastic transformation was observed in the Mo-exposed B-Mo cells, but not Si-exposed B-Si cells. Given that the magnitude of Mo effect was similar to the PM_{MTM}, despite its higher concentration than those presented in PM_{MTM}, it is likely that (i) some other elements could be involved in the PM_{MTM} effect and that (ii) such an effect arose from the synergistic effect of more than one component (e.g., Si and Mo).

Various carcinogenic properties representing the hallmarks of malignant cells were further assessed in this study. The transformed B-PM_{MTM} and B-Mo cells demonstrated excessive cell growth and altered cell cycle (Figures 3 and 4). To our knowledge, this is the first demonstration of the induction of cell proliferation by chronic low-dose PM exposure, although the inhibition of cell proliferation^{35,36} and induction of cytotoxicity^{37,38} by acute high-dose PMs from urban and industrial areas have previously been demonstrated. Interestingly, B-PM_{MTM} and B-Mo cells promoted cell cycle at different phases, possibly due to (i) the low content of Mo present in the PM_{MTM} that might not be sufficient to drive the cells to G2/M phase, and (ii) the PM_{MTM} proliferative effect was probably the result of components other than Mo or a synergistic effect of more than one component. Cell motility was also shown to increase significantly in the B-PM_{MTM} and B-Mo cells as compared to B-NTX cells (Figure 5), thus indicating their aggressive behaviors, which could be important in tumor progression.

The potential role of PM_{MTM} in lung carcinogenesis was further evaluated in vivo using a mouse xenograph model. Our results demonstrated that B-PM_{MTM} cells, although not directly tumor-inducing in mice, promoted tumor formation and metastasis of human lung cancer H460 cells (Figures 6 and 7). The limitation of our in vivo study is the relatively small number

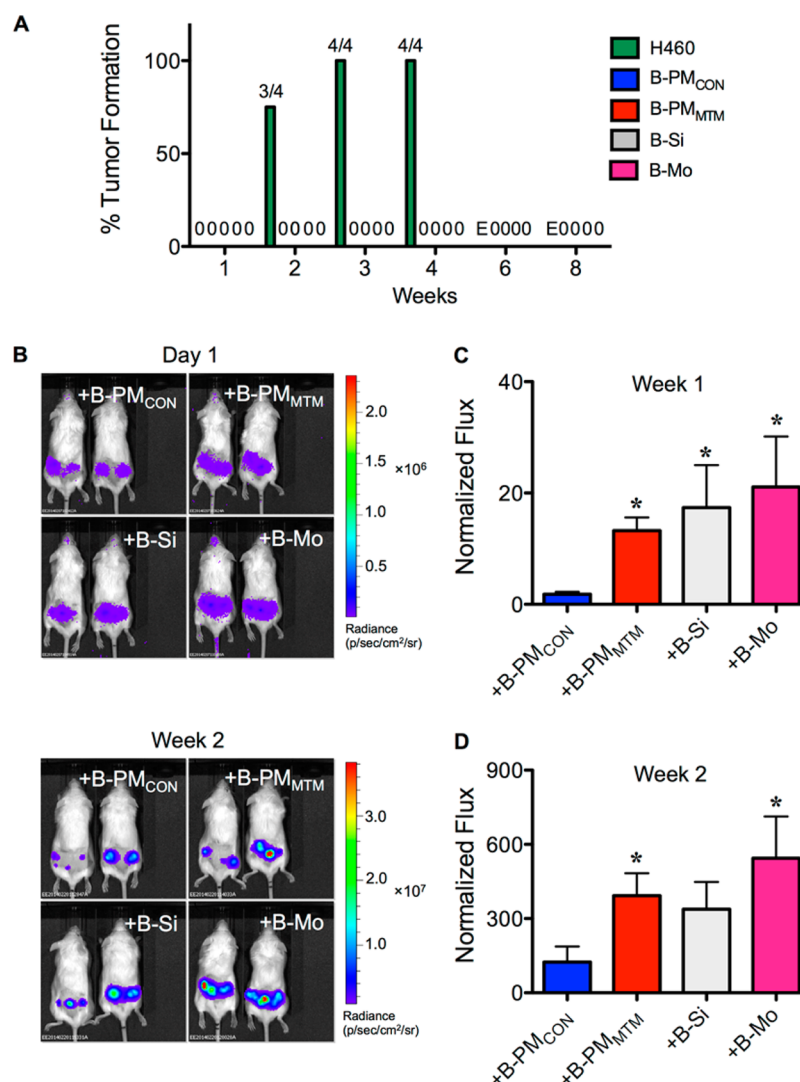


Figure 6. Chronic PM_{MTM} exposed cells promote tumor formation of human nonsmall cell lung cancer H460 cells in mice. (A) Growth kinetics of H460 or transformed B-PM_{CON}, B-PM_{MTM}, B-Si, and B-Mo cells (1×10^6 cells) when SC injected into the NSG mice alone. E indicates the end of experiment. (B) Transformed cells at the dose of 6×10^5 cells were coinjected with luciferase-labeled H460 cells at the dose of 3×10^5 cells (2:1 ratio) into the left and right flanks of NSG mice. Tumor formation was monitored weekly by IVIS bioluminescence imaging. Representative IVIS images of mice at day 1 and week 2 are shown. (C, D) Normalization of tumor bioluminescence signals at 1 (C) and 2 (D) weeks postinjection to their initial signal at day 1. Data are mean \pm SD ($n = 4$). * $P < 0.05$ (power >60%) vs H460 and B-PM_{CON} coinjection.

of animals per group, and given the high individual biological variation, we could not obtain statistical power >80%. Despite the low statistical power, we were still able to achieve statistical significance ($P < 0.05$). These results however are in good agreement with previous reports showing the hypermethylation of tumor suppressor p16 by PMs from urban areas, which could lead to cancer development³⁹ and the induction of lung carcinoma by Mo inhalation exposure in mice.⁴⁰

Taken together, the present study demonstrated that chronic exposure to PM_{MTM} induced neoplastic transformation of human bronchial epithelial cells with cancer-like properties. Although the data did not indicate tumor initiation by PM_{MTM}, the lung tumor promotion and progression by PM_{MTM} are a health concern as a cancer promoter. Our finding strengthens previous epidemiological studies linking MTM to increased incidence of lung cancer^{5,6,41} and supports prudent adoption of prevention strategies and exposure control for PM_{MTM}. As more than 60 000 cancer cases has been estimated to correlate with MTM activities in West Virginia,³¹ this finding on the cancer-promoting effect of

PM_{MTM} and related epidemiological data are crucial to raise public health awareness to reduce cancer risk. Our study also suggested that Mo could be one of the key inorganic elements responsible for the cancer-promoting effect of PM_{MTM}, although we could not rule out the involvement of organic elements as well as the synergistic effect of more than one element in the process. Finally, the chronic exposure model described in this study may be useful in further mechanistic studies and risk assessment of PM_{MTM} pathogenicity.

■ ASSOCIATED CONTENT

§ Supporting Information

Information includes the PM mass used in this study (Table S1), PM_{MTM} analysis of organic/elemental carbon, metals and sulfate (Table S2) and Supporting Information and Methods. This material is available free of charge via the Internet at <http://pubs.acs.org>.

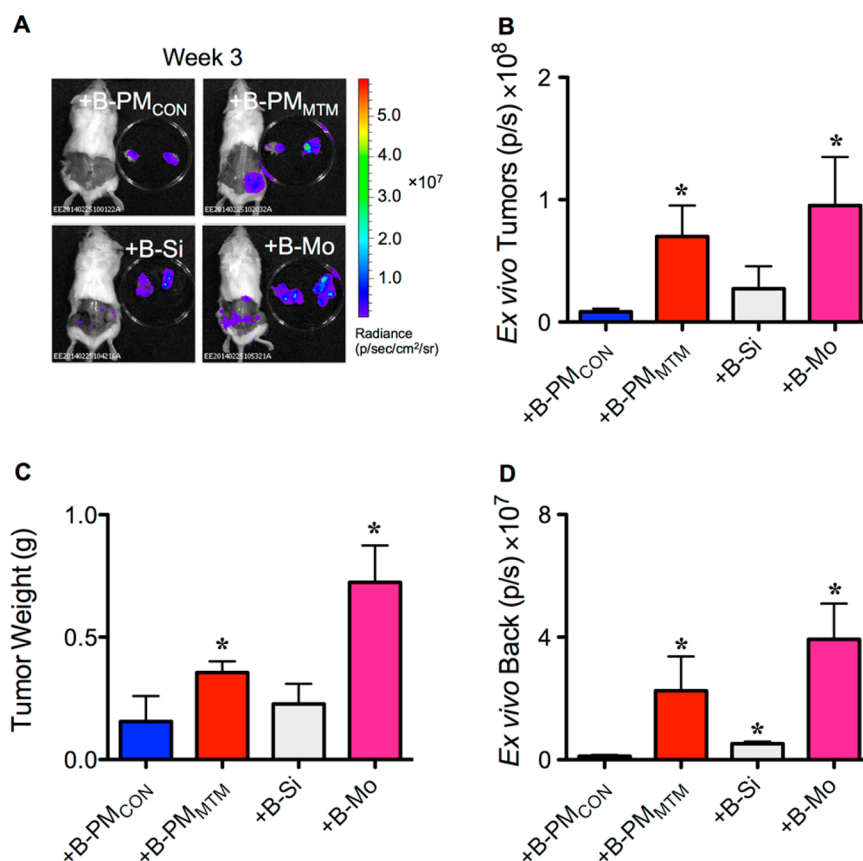


Figure 7. Analysis of ex vivo tumors of human lung cancer H460 cells. At 3 weeks postinjection, SC tumors were dissected from mice bearing H460 and B-PM_{CON}, B-PM_{MTM}, B-Si, or B-Mo cells. (A) Representative bioluminescence images of mice and SC tumors. (B) Quantitative analysis of bioluminescence signals from SC tumors. Data are mean \pm SD ($n = 4$). * $P < 0.05$ (power $> 60\%$) vs H460 and B-PM_{CON} coinjection. (C) The weight of dissected SC tumors. (D) Quantitative analysis of bioluminescence signals from the back of mice.

AUTHOR INFORMATION

Corresponding Author

*E-mail: suidjit@gmail.com. Tel: 304 293 1483. Fax: 304 293 2576.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The project described was supported by grants from the National Institute of General Medical Sciences [U54 GM104942 to S.L.] and National Institutes of Health [R01 HL095579 and R01 ES022968]. Flow cytometric analysis was performed in the West Virginia University Flow Cytometry Core Facility, which is supported in part by the National Institutes of Health [P30 GM103488]. Animal experiments were performed in the West Virginia University Animal Models and Imaging Facility, which is supported in part by the Mary Babb Randolph Cancer Center and National Institutes of Health [P20 RR016440, P30 RR032138/GM103488, S10 RR026378].

ABBREVIATIONS

MTM	mountaintop coal mining
PM	particulate matter
PM _{MTM}	MTM-derived PM
PM _{CON}	control PM
Si	silica
Mo	molybdenum
NTX	nontreatment

B-NTX	passage-matched control bronchial epithelial cells
B-PM _{CON}	chronic PM _{CON} -exposed bronchial epithelial cells
B-PM _{MTM}	chronic PM _{MTM} -exposed bronchial epithelial cells
B-Si	chronic Si-exposed bronchial epithelial cells
B-Mo	chronic Mo-exposed bronchial epithelial cells
NSG mice	NOD/SCID gamma mice
SC	subcutaneous

REFERENCES

- (1) Samel, J. M. Environmental causes of cancer: what do we know in 2003? *Chest* **2004**, *125* (5 Suppl), 80S–83S.
- (2) Samet, J. M.; Avila-Tang, E.; Boffetta, P.; Hannan, L. M.; Olivio-Marston, S.; Thun, M. J.; Rudin, C. M. Lung cancer in never smokers: clinical epidemiology and environmental risk factors. *Clin. Cancer Res.* **2009**, *15* (18), 5625–5645.
- (3) Lengerich, E. L.; Tucker, T. C.; Powell, R. K.; Colsher, P.; Lehman, E.; Ward, A. J.; Siedlecki, J. C.; Wyatt, S. W. Cancer incidence in Kentucky, Pennsylvania, and West Virginia: Disparities in Appalachia. *J. Rural Health* **2005**, *21* (1), 39–47.
- (4) Schoenberg, N. E.; Howell, B. M.; Fields, N. Community strategies to address cancer disparities in Appalachian Kentucky. *Fam. Community Health* **2012**, *35* (1), 31–43.
- (5) Hendryx, M.; O'Donnell, K.; Horn, K. Lung cancer mortality is elevated in coal-mining areas of Appalachia. *Lung Cancer* **2008**, *62* (1), 1–7.
- (6) Ahern, M.; Hendryx, M. Cancer mortality rates in Appalachian mountaintop coal mining areas. *J. Environ. Occup. Sci.* **2012**, *1* (2), 63–70.

- (7) Holzman, D. C. Mountaintop removal mining: Digging into community health concerns. *Environ. Health Perspect.* **2011**, *119* (11), A476–A483.
- (8) Annual coal report. U.S. Energy Information Administration Office of Oil, Gas, and Coal Supply Statistics, U.S. Department of Energy: Washington, DC, 2012. <http://www.eia.gov/coal/annual/pdf/acr.pdf>.
- (9) Ghose, M. K.; Majee, S. R. Characteristics of hazardous airborne dust around an Indian surface coal mining area. *Environ. Monit. Assess.* **2007**, *130* (1–3), 17–25.
- (10) Drew, R. T.; Witschi, H. Target organ toxicity: Lung introduction. *Environ. Health Perspect.* **1976**, *16*, 1–2.
- (11) Barrett, J. C. Mechanisms of multistep carcinogenesis and carcinogen risk assessment. *Environ. Health Perspect.* **1993**, *100*, 9–20.
- (12) Cohen, S. M.; Arnold, L. L. Chemical carcinogenesis. *Toxicol. Sci.* **2011**, *120* (S1), S76–S92.
- (13) Medan, D.; Luanpitpong, S.; Azad, N.; Wang, L.; Lu, Y.; Davis, M. E.; Jiang, B. H.; Barnett, J. B.; Guo, L.; Rojanasakul, Y. Malignant transformation and tumorigenesis of chromium (VI)-transformed lung cells and their inhibition by Bcl-2 knockdown. *PLoS One* **2012**, *7*, e37045.
- (14) Kurth, L. M.; McCawley, M.; Hendryx, M.; Lusk, S. Atmospheric particulate matter size distribution and concentration in West Virginia coal mining and non-mining areas. *J. Expo. Sci. Environ. Epidemiol.* **2014**, *24*, 405–411.
- (15) Knuckles, T. L.; Stapleton, P. A.; Minarchick, V. A.; Esch, L.; McCawley, M.; Hendryx, M.; Nurkiewicz, T. R. Air pollution particulate matter collected from an Appalachian mountaintop mining site induces microvascular dysfunction. *Microcirculation* **2013**, *20* (2), 158–169.
- (16) Luanpitpong, S.; Talbott, S. J.; Rojanasakul, Y.; Nimmannit, U.; Pongrakhananon, V.; Wang, L.; Chanvorachote, P. Regulation of lung cancer cell migration and invasion by reactive oxygen species and caveolin-1. *J. Biol. Chem.* **2010**, *285* (50), 38832–38840.
- (17) Basset, G.; Crone, C.; Saumon, G. Fluid absorption by rat lung *in situ*: Pathways for sodium entry in the luminal membrane of alveolar epithelium. *J. Physiol.* **1987**, *384*, 325–345.
- (18) Rivero, D. H.; Soares, S. R.; Lorenzi-Filho, G.; Saiki, M.; Godleski, J. J.; Antonangelo, L.; Dolnikoff, M.; Saldiva, P. H. Acute cardiopulmonary alterations induced by fine particulate matter of Sao Paulo, Brazil. *Toxicol. Sci.* **2005**, *82* (2), 898–905.
- (19) U.S. EPA. Inhalation rates. *Exposure Factors Handbook 2011 ed. (Final)*; U.S. Environmental Protection Agency: Washington, DC, 2011. EPA/600/R-09/052F, 2011.
- (20) Liu, B.; Wang, L.; Shen, L. L.; Shen, M. Z.; Guo, X. D.; Wang, T.; Liang, Q. C.; Wang, C.; Zheng, J.; Li, Y.; Jia, L. T.; Zhang, H.; Gao, G. D. RNAi-mediated inhibition of presenilin 2 inhibits glioma cell growth and invasion and is involved in the regulation of Nrg1/ErbB signaling. *Neuro. Oncol.* **2012**, *14* (8), 994–1006.
- (21) Luanpitpong, S.; Wang, L.; Castranova, V.; Rojanasakul, Y. Induction of stem-like cells with malignant properties by chronic exposure of human lung epithelial cells to single-walled carbon nanotubes. *Part. Fibre Toxicol.* **2014**, *11*, 1–17.
- (22) Luanpitpong, S.; Wang, L.; Stueckle, T.; Tse, W.; Chen, Y. C.; Rojanasakul, Y. Caveolin-1 regulates p53 inactivation and stem-like induction of carbon nanotube-driven lung tumorigenesis. *Oncotarget* **2014**, *5* (11), 3541–3554.
- (23) Gjoerup, O. Polyomaviruses and cancer. In *Cancer Associated Viruses*; Robertson, E. S., Ed.; Springer: New York, 2012.
- (24) Hanahan, D.; Weinberg, R. A. The hallmarks of cancer. *Cell* **2000**, *100*, 57–70.
- (25) Evan, G. I.; Vousden, K. H. Proliferation, cell cycle and apoptosis in cancer. *Nature* **2001**, *411*, 342–348.
- (26) Friedl, P.; Wolf, K. Tumour-cell invasion and migration: Diversity and escape mechanisms. *Nat. Rev. Cancer* **2003**, *3*, 362–374.
- (27) Friedl, P.; Gilmour, D. Collective cell migration in morphogenesis, regeneration and cancer. *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 445–457.
- (28) Vincent, T. L.; Gatenby, R. A. An evolutionary model for initiation, promotion, and progression of carcinogenesis. *Int. J. Oncol.* **2008**, *32* (4), 729–737.
- (29) Dragan, Y. P.; Sargent, L.; Xu, Y. D.; Xu, Y. H.; Pilot, H. C. The initiation-promotion-progression model of rat hepatocarcinogenesis. *Exp. Biol. Med.* **1993**, *202*, 16–24.
- (30) Sargent, L. M.; Porter, D. W.; Staska, L. M.; Hubbs, A. F.; Lowry, D. T.; Battelli, L.; Siegrist, K. J.; Kashon, M. L.; Mercer, R. R.; Bauer, A. K.; Chen, B. T.; Salisbury, J. L.; Frazer, D.; McKinney, W.; Andrew, M.; Tsuruoka, S.; Endo, M.; Fluharty, K. L.; Castranova, V.; Reynolds, S. H. Promotion of lung adenocarcinoma following inhalation exposure to multi-walled carbon nanotubes. *Part. Fibre Toxicol.* **2014**, *11*, 3.
- (31) Hendryx, M.; Wolfe, L.; Luo, J.; Webb, B. Self-reported cancer rates in two rural areas of West Virginia with and without mountaintop coal mining. *J. Community Health* **2012**, *37* (2), 320–327.
- (32) Lippmann, M.; Yeates, D. B.; Albert, R. E. Deposition, retention, and clearance of inhaled particles. *Br. J. Ind. Med.* **1980**, *37*, 337–362.
- (33) Ke, Y.; Reddel, R. R.; Gerwin, B. I.; Miyashita, M.; McMenamin, M.; Lechner, J. F.; Harris, C. C. Human bronchial epithelial cells with integrated SV40 virus T antigen genes retain the ability to undergo squamous differentiation. *Differentiation* **1988**, *38*, 60–66.
- (34) Stewart, C. E.; Torr, E. E.; Mohd Jamili, N. H.; Bosquillon, C.; Sayers, I. Evaluation of differentiated human bronchial epithelial cell culture systems for asthma research. *J. Allergy* **2012**, *2012*, 943982.
- (35) Longhin, E.; Holme, J. A.; Gutzkow, K. B.; Arlt, V. M.; Kucab, J. E.; Camatini, M.; Gualtieri, M. Cell cycle alterations induced by urban PM_{2.5} in bronchial epithelial cells: characterization of the process and possible mechanisms involved. *Part. Fibre Toxicol.* **2013**, *10*, 63.
- (36) Zhang, J.; Ghio, A. J.; Gao, M.; Wei, K.; Rosen, G. D.; Upadhyay, D. Ambient particulate matter induces alveolar epithelial cell cycle arrest: Role of G1 cyclins. *FEBS Lett.* **2007**, *581* (27), S315–S320.
- (37) Garcon, G.; Dagher, Z.; Zerimech, F.; Ledoux, F.; Courcot, D.; Aboukais, A.; Puskaric, E.; Shirali, P. Dunkerque City air pollution particulate matter-induced cytotoxicity, oxidative stress and inflammation in human epithelial lung cells (L132) in culture. *Toxicol. In Vitro* **2006**, *20* (4), 519–528.
- (38) Frampton, M. W.; Ghio, A. J.; Samet, J. M.; Carson, J. L.; Carter, J. D.; Devlin, R. B. Effects of aqueous extracts of PM₁₀ filters from the Utah Valley on human airway epithelial cells. *Am. J. Physiol.* **1999**, *277* (5 Pt 1), L960–L970.
- (39) Soberanes, S.; Gonzalez, A.; Urich, D.; Chiarella, S. E.; Radigan, K. A.; Osornio-Vargas, A.; Joseph, J.; Kalyanaraman, B.; Ridge, K. M. Particulate matter air pollution induces hypermethylation of the p16 promoter via a mitochondrial ROS-JNK-DNMT1 pathway. *Sci. Rep.* **2012**, *2*, 275.
- (40) Chan, P. C.; Herbert, R. A.; Roycroft, J. H.; Haseman, J. K.; Grumbein, S. L.; Miller, R. A.; Chou, B. J. Lung tumor induction by inhalation exposure to molybdenum trioxide in rats and mice. *Toxicol. Sci.* **1998**, *45* (1), 58–65.
- (41) Hendryx, M.; Fedorko, E.; Anesetti-Rothermel, A. A geographical information system-based analysis of cancer mortality and population exposure to coal mining activities in West Virginia, United States of America. *Geospat. Health* **2010**, *4* (2), 243–256.