Activation of HGF/c-Met signaling by ultrafine carbon particles and its contribution to alveolar type II cell proliferation

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Chang CC, Chiu JC, Chen SL, Huang HC, Chiu HF, Lin BH, Yang CY. Activation of HGF/c-Met signaling by ultrafine carbon particles and its contribution to alveolar type II cell proliferation. Am J Physiol Lung Cell Mol Physiol 302: L755–L763, 2012. First published January 13, 2012; doi:10.1152/ajplung.00350.2011.—Hepatocyte growth factor (HGF) is a potent mitogen and motogen for various epithelial cells. The present study aimed to explore the role of HGF and c-Met receptor in ultrafine carbon particle–induced type II epithelial (type II) cell proliferation. ICR mice were intratracheally instilled with 100 μg ultrafine carbon black (ufCB) and killed at 21, 48, and 72 days postexposure to examine type II cell proliferation, HGF release, and c-Met activation. In vivo and in vitro applications of neutralizing anti-HGF antibody were used to investigate the causal role of HGF in cell proliferation. The Met kinase inhibitor SU11274 and extracellular signal-regulated kinase 1/2 (ERK1/2) inhibitor PD98059 were used to delineate the involvement of c-Met/ERK1/2 in rat L2 pulmonary epithelial cell proliferation. The results demonstrated that in vivo exposure to 100 μg ufCB caused increased HGF in bronchoalveolar lavage fluid, as well as increased HGF production, c-Met phosphorylation, and cell proliferation in type II cells. In vitro study revealed that ufCB caused a dose-dependent increase in HGF release, c-Met phosphorylation, and cell proliferation. Importantly, treatment with the neutralizing anti-HGF antibody significantly blocked ufCB–induced proliferation in vivo and in vitro type II cell proliferation. Moreover, SU11274 and PD98059 significantly reduced ufCB–increased L2 cell proliferation. Results from Western blotting demonstrated that SU11274 successfully suppressed ufCB–induced phosphorylation of c-Met and ERK1/2. In summary, the activation of HGF/c-Met signaling is a major pathway involved in ufCB–induced type II cell proliferation.

CARBON-CENTERED ULTRAFINE PARTICLES (≤0.1 μm in diameter) comprise the majority of fine particulate matters and are postulated to be responsible for the association between ambient air particles and the development of respiratory diseases (13, 18). Studies (10, 22, 36) have indicated that ultrafine particles can cause a greater pulmonary inflammatory response, injury, and epithelial cell proliferation than larger particles of the same mass can. In vitro studies (33, 35) have shown that exposure to ultrafine particles causes the activation of EGF receptor (EGFR) and extracellular signal–regulated kinase 1/2 (ERK1/2), resulting in pulmonary cell proliferation.

Hepatocyte growth factor (HGF) is a potent mitogen and motogen for various epithelial cells (31, 34). HGF is produced following HCl- or bleomycin-induced acute lung injury and plays a role in pulmonary epithelial cell regeneration (1, 40). Studies (14, 26) have also demonstrated that HGF is responsible for most growth-promoting activity for type II cells, compared with EGF, tumor growth factor–α (TGF-α), acidic fibroblast growth factor, and keratinocyte growth factor. To date, the role of HGF in pulmonary epithelial cell proliferation after exposure to environmental particulate matter, in particular ultrafine particles, has not been reported.

HGF exerts its effects through c-Met receptors, which are mainly expressed in cells with an epithelial or endothelial origin. The c–met gene was initially identified as an oncogene (27, 31). Incubation with HGF results in the activation of c-Met and increase of proliferation in lung adenocarcinoma cells and isolated type II cells and stimulation of ERK1/2 phosphorylation in lung adenocarcinoma cells (6). Activated ERK1/2 can induce cell proliferation by phosphorylating nucleotide synthesis enzymes or enhancing activator protein–1 activity (4). Therefore, c-Met signaling through ERK1/2 may function to stimulate pulmonary epithelial cell proliferation.

The purpose of this study was to explore the role of HGF/c-Met in alveolar epithelial cell proliferation after ultrafine carbon particle exposure. Accordingly, we first examined the proliferation of type II cells, release of HGF, and phosphorylation of c-Met following ultrafine carbon particle exposure in mice. A neutralizing anti-HGF antibody was used to evaluate the causal role of HGF in ultrafine carbon particle–induced type II cell proliferation in vivo and in vitro. Western blotting using the c-Met inhibitor SU11274 and the ERK1/2 inhibitor PD98059 provided mechanistic evidence for the involvement of c-Met/ERK1/2 signaling in ultrafine carbon particle–induced type II cell proliferation.

MATERIALS AND METHODS

Particle preparation. Ultrafine carbon black (ufCB; Printex 90; 14 nm in diameter with a surface area of 253.9 m2/g; Degussa, Frankfurt, Germany) and fine carbon black (fCB; N990, 250 nm in diameter with a surface area of 7.9 m2/g; Degussa) were weighed, ground, and suspended in PBS (pH 7.4). The suspension was sonicated (W-250; Branson, Danbury, CT) for 20 min (20 W) at a concentration of 1 μg/μL. Immediately after sonication, particle size distribution was determined by dynamic light scattering (DLS; Delsa Nano; Beckman Coulter, Brea, CA).

Animals. Male ICR mice were purchased from the animal center at National Cheng Kung University (Tainan, Taiwan). All animal experiments were conducted according to the American Physiological Society’s Guiding Principles in the Care and Use of Vertebrate Animals in Research and Training. The procedures were approved by Public Health, Kaohsiung Medical Univ., No. 100, Shi-Chuan Rd, Kaohsiung City, Taiwan 807 (e-mail: chunyuh@kmu.edu.tw).

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overnight at room temperature. After blocking, 50 g/ml of 25 cm H2O for 5 min. The specimens were then sub-
merged in 10% formaldehyde at 4°C for 24 h. After reaction with 75 g/ml of neutralizing anti-HGF antibody (AF294NA, R&D Systems), 0.01 M Na2Si1227 (Calbiochem, La Jolla, CA), or 0.01 M ERK1/2 inhibitor PD98059 (Cell Signaling Technology) were added to the medium 1 h before treatment with uCB. BrdU (10 M) was added 2 h before the end of treatment. After the cells were washed, peroxi-
dase-labeled anti-BrdU antibody (1:100) was then added for 45 min, and the absorbance was measured at 450 nm using a microplate reader (OpsysMR; DYNEX).

Western blot analysis. Cultured cells were treated with lysis buffer containing 1% Nonidet P-40, 0.05% sodium deoxycholate, 1 mM Na3VO4, 1 mM NaF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride. The supernatant was collected, and equal amounts of cell lysates (200 μg) were loaded to each lane of an 8% sodium dodecyl sulfate polyacrylamide gel. After electrophoresis and protein transfer, the membrane was exposed to light-
ning film (Kodak, Boston, MA) to visualize the bands. A com-
puterized image analyzer was then used to apply densitometry to quantify the results (GelDoc-IT System; UVP, Upland, CA).

Primary antibodies included c-Met (1:500; no. 3127; Cell Signaling Technology), p-c-Met (pThy1234/1235; 1:500; no. 3129; Cell Signaling Technology), ERK1/2 (1:1,000; no. 9102; Cell Signaling Technology), phospho-ERK1/2 (pThr202/Tyr204; p-ERK1/2; 1:1,000; no. 4377; Cell Signaling Technology), and β-actin (1:5,000; Chemicon International, Temecula, CA).

In vitro incorporation of bromode-
thyymidine (BrdU) was assessed by fluorescence microscopy (Olympus). Images were captured using a CoolSNAP camera (Photometrics, Tucson, AZ) and analyzed with MetaMorph 7.5 software (Universal Imaging, West Chester, PA).

For quantification of the percentage of SP-positive type II cells that stained positive for PCNA (PCNA labeling index), 8 randomly spaced sections from each animal and each with ≥20 nonoverlapping high power fields were examined.

In vitro cell proliferation assay. In vitro incorporation of bromode-
thyymidine (BrdU) was assayed as a measure of cell proliferation (Amersham Pharmacia Biotech, Buckinghamshire, UK). At a density of 5 × 104 cells per well, L2 cells were plated in 100 μl Ham’s F-12 medium with 0.5% FBS for 24 h before treatment with 50 μg/ml uCB, 100 μg/ml uCB, 20 ng/ml HGF, or 100 μg/ml fCB for 24 h. To study the role of HGF and c-Met signaling on cell proliferation, 10 μg/ml of neutralizing anti-HGF antibody (AF294NA, R&D Systems), 0.01 M Na2Si1227 (Calbiochem, La Jolla, CA), or 0.01 M ERK1/2 inhibitor PD98059 (Cell Signaling Technology) were added to the medium 1 h before treatment with uCB. BrdU (10 M) was added 2 h before the end of treatment. After the cells were washed, peroxi-
dase-labeled anti-BrdU antibody (1:100) was then added for 45 min, and the absorbance was measured at 450 nm using a microplate reader (OpsysMR; DYNEX).

RESULTS

Particle size distribution. Sonication of 100 μg uCB in PBS resulted in reasonably dispersed uCB with a mean diameter of 104.7 nm ± 27.0 SD, as measured by the DLS technique (Fig. 1A). The mean diameter of fCB dispersed in PBS was 314.0 ± 77.2 nm SD (Fig. 1B).

Effects of uCB exposure on leukocyte infiltration, total proteins, LDH activity, and HGF release. The results from a dose-dependent study demonstrated that the instilled dose of 100 μg uCB caused significantly higher HGF release than did 50 μg (95 ± 2.4 vs. 74 ± 3.5 ng/ml, respectively; P < 0.01) at 21 h postexposure. As a result, we selected the dose of 100 μg per mouse for the subsequent experiments.

BAL leukocytes with differential counts, total proteins, and LDH were measured as indicators of pulmonary inflammation and injury after uCB exposure. When compared with PBS controls, uCB induced a significant influx of BAL neutrophils at 21, 48, and 72 h postexposure, while the extent of neutrophil influx at 72 h was less prominent. The number of macrophages increased significantly at 48 and 72 h after exposure to uCB...
Total proteins in BAL fluid were significantly increased at 21, 48, and 72 h in the ufCB group, compared with the PBS group (Fig. 2B). LDH activity was significantly increased in BAL fluid following exposure to ufCB at each time point (Fig. 2C). Moreover, there were significant increases of HGF in BAL fluid at 21, 48, and 72 h postexposure in the ufCB group, compared with those in the PBS group. HGF reached the maximal level at 48 h and remained significantly elevated at 72 h (Fig. 2D).

**Cell proliferation, c-Met activation, and HGF production in type II cells following ufCB exposure.** We next examined whether elevated HGF contributed to type II cell proliferation. Figure 3 presents the immunofluorescence analysis of mouse lungs 48 h after ufCB exposure (100 μg/mouse). Type II cell proliferation occurred within 21 h of ufCB exposure (data not shown). At 48 h, more type II cells were positively stained with anti-PCNA antibody in the ufCB exposure group, compared with those in the PBS group (Fig. 3B). Treatment with the neutralizing anti-HGF antibody reduced ufCB-induced type II cell proliferation (Fig. 3C). The normal IgG control did not show any effect on type II cell proliferation (Fig. 3D). Results in Fig. 3E show the percentage of SPC-positive type II cells that stained positive for PCNA, expressed as PCNA labeling index, in each treatment group. The PCNA labeling index was significantly higher in the ufCB-exposure group, compared with that in the PBS group (19.02 ± 0.31 vs. 0.73 ± 0.31%, respectively; P < 0.01). In addition, the PCNA labeling index was significantly lower in the ufCB plus neutralizing anti-HGF antibody group, compared with that of the ufCB group (1.75 ± 0.17 vs. 19.02 ± 0.31%, respectively; P < 0.01).

Immunohistochemistry studies indicated that the increase in the number of type II cells with immunoreactive p-c-Met (Fig. 2A). Total proteins in BAL fluid were significantly increased at 21, 48, and 72 h in the ufCB group, compared with the PBS group (Fig. 2B). LDH activity was significantly increased in BAL fluid following exposure to ufCB at each time point (Fig. 2C). Moreover, there were significant increases of HGF in BAL fluid at 21, 48, and 72 h postexposure in the ufCB group, compared with those in the PBS group. HGF reached the maximal level at 48 h and remained significantly elevated at 72 h (Fig. 2D).

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became apparent at 8 h after exposure in the ufCB group, compared with the PBS group (Fig. 4, A and B). The number of cells with immunoreactive total c-Met did not differ between the PBS and ufCB groups (Fig. 4, C and D).

To explore the possibility that ufCB exposure caused autocrine HGF production in type II cells, we performed the immunofluorescent staining for HGF. The results demonstrated that HGF production was clearly visible in type II cells at 8 h postexposure to ufCB (Fig. 4 E). In the PBS group, there was no HGF staining in type II cells (Fig. 4 F).

In vitro study of ufCB-induced cell proliferation. To clearly establish the relationship between HGF/c-Met activation and type II cell proliferation, we carried out an in vitro cell proliferation study with neutralizing anti-HGF antibody, the c-Met inhibitor SU11274, and the ERK1/2 inhibitor PD98059.

Exposure to ufCB caused dose- and time-dependent increases in L2 cell proliferation (Fig. 5A). The neutralizing anti-HGF antibody at 5 or 10 μg/ml significantly suppressed ufCB-induced cell proliferation (Fig. 5B), while normal goat IgG had no effect. The c-Met inhibitor SU11274 was used to examine the role of c-Met in ufCB-stimulated cell proliferation. SU11274 at 0.01, 0.05, or 0.1 μM dose-dependently reduced L2 cell proliferation incured by ufCB exposure. Furthermore, the stimulatory effect of ufCB on cell proliferation was significantly inhibited by the addition of 50 or 100 μM PD98059, a specific ERK1/2 inhibitor (Fig. 5C). Therefore, these data indicate that the HGF-c-Met-ERK1/2 signaling cascade plays an important role in ufCB-induced cell proliferation.

ufCB-induced activation of c-met and ERK1/2 in L2 cells. To provide mechanistic evidence for the involvement of c-Met-ERK1/2 signaling in ufCB-induced cell proliferation, Western blot analysis was used to investigate the phosphorylation of c-Met/ERK1/2 following ufCB exposure.

A time-dependent study on the activation of c-Met revealed that 100 μg/ml ufCB caused the peak phosphorylation of c-Met at 8 h postexposure (Fig. 6A). At this time point, ufCB triggered a dose-dependent production of HGF in L2 cells. In contrast, fCB exposure did not cause increased HGF production, compared with PBS (Fig. 6B).

Figure 7 reveals that the exposure of L2 cells to ufCB resulted in significant dose-dependent increases of c-Met phosphorylation. Pretreatment with SU11274 at 0.05 and 0.1 μM

Fig. 3. Neutralizing anti-HGF antibody significantly reduced ufCB (100 μg/mouse)-increased type II cell proliferation at 48 h postexposure in ICR mice. Representative immunofluorescent staining: PBS control: all were surfactant protein C (SPC)-positive (green) type II cells (A); ufCB group: increased proliferating cell nuclear antigen (PCNA) staining (red) in type II cells (B); ufCB plus neutralizing anti-HGF group: decreased PCNA staining in type II cells (C); and ufCB plus normal IgG control group: no obvious effect on the number of PCNA-positive type II cells (D). Quantitative results of the number of type II cells stained positively for PCNA: PCNA labeling index in ufCB exposure group is 19.02 ± 0.31% (E). Neutralizing anti-HGF antibody significantly reduces the PCNA labeling index to 1.75 ± 0.17%. Value of each point is expressed as a means ± SE of 4 mice. *Significantly different from the PBS group or between groups (P < 0.01). Scale bar = 20 μm.
led to significant dose-dependent decreases in ufCB-induced c-Met phosphorylation.

The role of c-Met phosphorylation in the activation of ERK1/2 is also demonstrated in Fig. 7A. Similar to c-Met, ERK1/2 was phosphorylated in a dose-dependent manner following ufCB exposure. Importantly, SU11274 at 0.05 and 0.1 μM significantly and dose dependently decreased ufCB-induced phosphorylation of ERK1/2.

Taken together, ufCB exposure led to the autocrine production of HGF and activation of c-Met receptor kinase, which in turn phosphorylated ERK1/2.

DISCUSSION

In this work, our aim was to determine the role of HGF and its receptor c-Met are responsible for pulmonary epithelial cell proliferation after ultrafine carbon particle exposure using in vivo and in vitro experiments. We demonstrated that in vivo exposure to ufCB caused pulmonary injury with increased HGF release in BAL fluid, HGF production, c-Met activation, and cell proliferation in type II cells. Most significantly, the neutralizing anti-HGF antibody blocked ufCB-induced type II cell proliferation. An in vitro study provided evidence that ufCB dose dependently phosphorylated c-Met/ERK1/2 and caused cell proliferation. Su11274 significantly suppressed ufCB-induced c-Met activation, ERK1/2 phosphorylation, and cell proliferation. To the best of our knowledge, this is the first study to demonstrate that ufCB exposure activates HGF/c-Met/ERK1/2, which resulted in cell proliferation.

This study demonstrates the causal role of HGF in type II cell proliferation in response to in vivo ufCB-induced pulmonary injury (Fig. 3). In this context, type II cell proliferation may reflect the need for timely repair of injured pulmonary epithelium. The integrity of pulmonary epithelial cells is important for maintaining pulmonary function and helps prevent the development of adverse outcomes. In bleomycin-induced lung injury, exogenous recombinant HGF enhances the repair process and attenuates fibrosis formation by increasing alveolar epithelial cell proliferation and fibrinolytic capacity (7). Extensive alveolar epithelial cell apoptosis leads to lung emphysematous changes (2). In vivo HGF gene transfer has been shown to ameliorate alveolar wall apoptosis, promote vascular regeneration, and improve pulmonary function in elastase-treated mice (25). In human emphysematous patients, decreased production of HGF by fibroblasts was demonstrated, suggesting that insufficient alveolar repair may contribute to the development of emphysema (19).

Adequate fibrinolytic activity around the migrating cells or injury sites is required for proliferating cells to migrate and cover the denuded area. HGF has been shown to significantly increase the expression of urokinase plasminogen activator (uPA) and generate surface plasmin on alveolar epithelial cells (7), both of which can degrade the extracellular matrix. This
process allows the cell to escape its binding to the extracellular matrix and migrate. In addition, the binding of uPA to its receptor uPAR and the interaction of uPA/uPAR with integrin and matrix plasminogen activator inhibitor-1 have been shown to facilitate the migration of epithelial cells (29). Research has also shown that HGF induces the expression of uPA and uPAR, and cell motility acts through a signaling pathway involving c-Met/ERK1/2 in MDCK cells (39). Therefore, the ufCB-activated HGF/c-Met/ERK1/2 signaling pathway may also account for the migration of type II cells, facilitating the repair of injured epithelium.

Previous work (35) with airway epithelium revealed that ufCB at 50 \( \mu \text{g/mL} \) induced the activation of EGFR and ERK1/2, as well as cell proliferation. The peak release of membrane HB-EGF was at 20 min. At 10 \( \mu \text{g/mL} \), ufCB induced significant and biphasic activation of EGFR at 2 min and at 120–480 min, significant phosphorylation of ERK1/2 at 8 h, and increased cell proliferation at 24 h in rat RLE-6TN type II cells (33). HGF is an autocrine factor for human normal bronchial epithelial cells in culture (38). HGF mRNA is detectable in isolated type II cells and is significantly increased after nicotine exposure, starting at 6 h postexposure (5). As shown in Fig. 6, ufCB at 100 \( \mu \text{g/mL} \) caused significant HGF production and peak c-Met activation at 8 h. It is reasonable to postulate that the increased HGF and EGF-like ligand may act interactively on type II cell proliferation. In normal rat hepatocytes, HGF was shown to induce expression of the EGFR ligand TGF-\( \alpha \). A neutralizing antibody against TGF-\( \alpha \) or anti-sense TGF-\( \alpha \) mRNA oligonucleotides reduced the mitogenic effect of HGF by 30–40\% (37). HGF and EGF act additively on DNA synthesis through the activation of the Ras/MAPK pathway in...
normal rat hepatocytes (17). Further studies are warranted to delineate the differential roles of EGFR and c-Met in the activation of MAPK and type II cell proliferation following ultrafine carbon particle exposure.

The coexpression of c-Met and HGF in the same cell can efficiently transform the cell via an autocrine stimulatory loop (23). Pulmonary adenocarcinomas overexpress c-Met more than squamous cell carcinomas do. The level of c-met mRNA is three times higher in adenocarcinomas than in normal lung tissues (12). The ki-67 proliferation index and both stromal HGF and intratumoral c-Met expression are closely associated in nonsmall cell lung cancer (15). Overexpression of HGF and/or the c-met gene in transgenic mice contributes to oncogenic transformation (3, 11). Moreover, transgenic overexpression of HGF in airway epithelial cells was shown to increase the risk of lung tumors following tobacco exposure (28). These earlier findings suggest that the HGF/c-Met pathway may play a significant role in the development of lung carcinoma. The fact that ultrafine particle exposure causes the activation of HGF/c-Met signaling in pulmonary epithelial cells may have significant implications in the transformation of these cells in response to long-term exposure to such particulate matter.

There are several limitations to this study, and therefore, the extrapolation of our results requires caution. First, we dispersed ultrafine carbon particles in PBS, instead of a biocompatible medium with dipalmitoyl phosphotidylcholine and serum albumin. However, the postsonication particle size of ufCB in PBS [104.7 ± 27.0 nm (1 μg/ml; 20 W, 20 min)] is compatible with that in BAL fluid [131 ± 4 nm (0.5 μg/ml; 10 W, 30 min)] or in biocompatible medium [93 ± 4 nm (0.5 μg/ml); Ref. 20]. It is different from the mean diameter of 281 nm for the CB nanoparticle (13 nm in diameter), prepared as 2 mg/ml stock (60 W, 3 min) and diluted in PBS (1 mg/ml, final concentration) before analyzed by DLS (8). The difference in the preparation of ultrafine carbon particles, including the choice of suspension media, particle concentration, and sonication power, may contribute to the variability in the size distribution of ultrafine carbon particles. Ultrafine carbon particles are prone to aggregation when suspended in PBS. However, the instilled ultrafine carbon particles would gain access to and mix with BAL fluid during the respiratory cycle. Ideally, in situ measurements of the nanoparticle dispersion state are more logical and important, compared with the assessment of nanoparticle dispersion state in medium, although it can be very difficult to measure (21). The surface area threshold dose for an inflammatory response of the instilled nanoparticles suspended in PBS is ~20 cm² in mice (30), and the alveolar surface area of mice is 0.05 m² (32). Therefore, the particle surface area threshold dose for inflammatory response is 0.04 cm²/cm³ (particle surface area per unit alveolar surface area). ufCB suspended in fresh rat BAL fluid has been found to induce an inflammatory response in rats at a particle surface dose of 0.03413 cm²/cm³ (24). Therefore, even though there might be differences in the pulmonary response to ultrafine particles suspended in different media, they would likely be small and not affect the validity of our study, which aimed at investigating the molecular mechanism of ultrafine particle-induced type II cell proliferation. Second, to visualize in vivo type II cell proliferation, we used an instilled dose of 0.5 cm²/cm³, which is >12 times the particle surface area threshold dose for an inflammatory response. The Occupational Safety and Health Administration exposure limit for respirable CB is 3.5 mg/m³. Even with 3.5 μg/cm³ as the exposure limit for ufCB, it would take 476.2 days for a mouse to inhale 100 μg ufCB (3.5 μg/cm³ × mouse ventilation volume 0.06 m³/day × A days = 100 μg, and A = 476.2 days), if exposed throughout the day for 1 wk. In future investigations, it will be important to establish an effective inhalation dose for ultrafine carbon particles, which can induce oncogene activation and cell proliferation. Third, the in vitro surface area threshold dose, expressed as the particle surface area per unit of cellular surface area, for the ufCB-induced inflammatory response in pulmonary epithelial cells, is ~10 cm²/cm³ (16). Starting at 50 μg/ml, equivalent to 12.7 cm²/cm³, which is slightly higher than the surface area threshold dose for an inflammatory response, ufCB is capable of activating c-Met (Fig. 7) and inducing L2 type II cell proliferation (Fig. 5). Theoretically, the in vitro cellular exposure system, compared with the in vivo one, is more prone to be affected by the dispersion state of the ultrafine carbon particles at and during the time of exposure. A comprehensive, comparative study is needed to address this aspect.

In conclusion, our present findings support the hypothesis that ultrafine carbon particles can stimulate pulmonary epithe-
HGF/C-MET, UCB-INDUCED CELL PROLIFERATION

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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