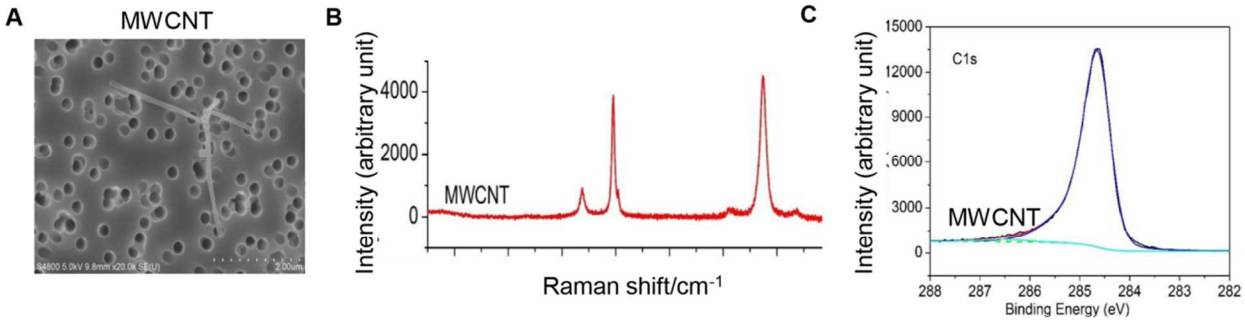
SUPPORTING INFORMATION

Supporting information includes Supplementary Figure S1, Supplementary Figure S2, Supplementary Figure S3 and Supplementary Experimental Methods.

SUPPLEMENTARY FIGURE



D **Characteristics of MWCNT**

Dimension	Hydrodynamic size	Zeta potential	Composition	ID/IG
Length: 5.28 ± 2.07 mm Width: 79.7 ± 19.4 nm	84.2 ± 21.6 nm	In H_2O: -27.6±0.306 mV In FBM: -10.5±0.53 mV	C:99%, Na: 0.41% Fe:0.32	0.232

Figure S1. **Characterization of MWCNTs.** (**A**) Transmission electron micrograph of MWCNTs. (**B**) Raman spectra of MWCNTs. (**C**) X-ray photoelectron spectroscopy (XPS) survey scan spectrum of MWCNTs. (**D**) Characteristics of MWCNTs.

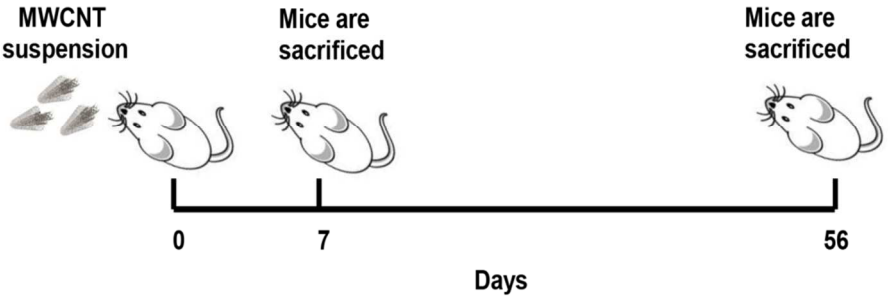


Figure S2. Schematic diagram of MWCNT oropharyngeal aspiration in C57BL/6 J mice.

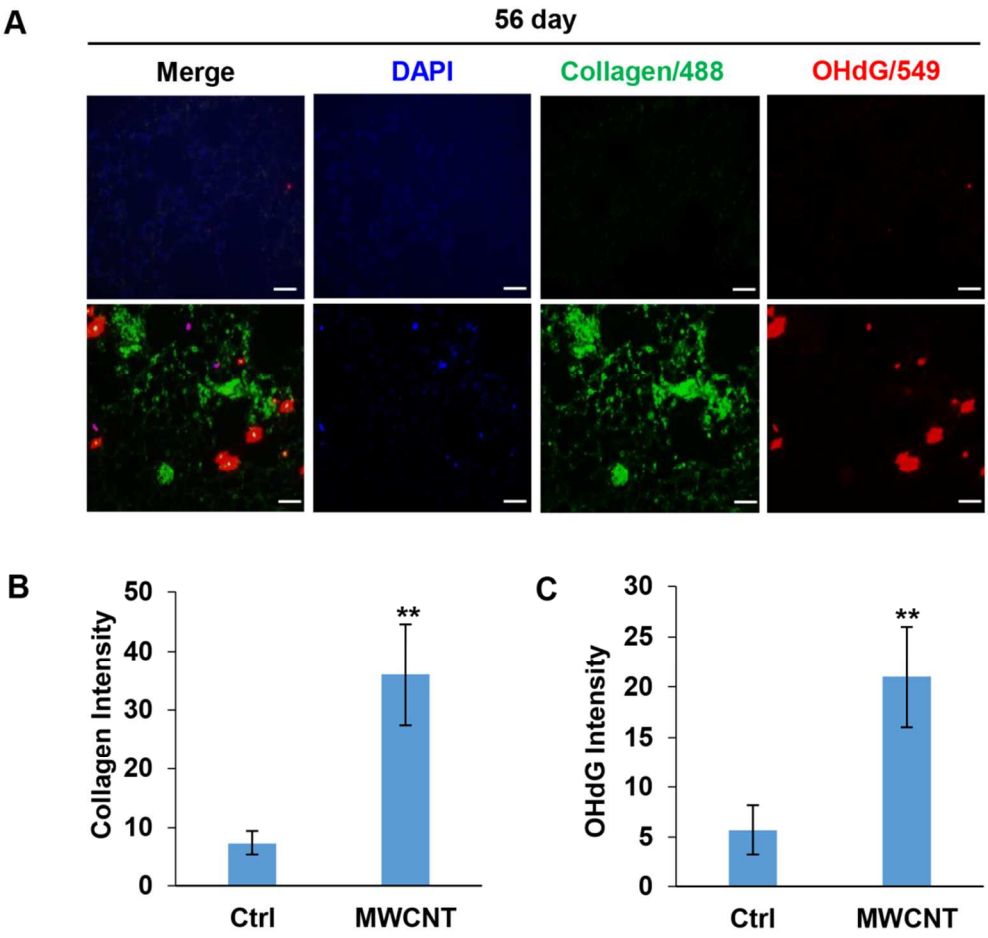


Figure S3. (**A**) Immunofluorescence staining and quantification of collagen and OHdG in mouse lungs at 56 days. (**B & C**) Quantitation of staining was performed using an image analysis software. Scale bar is 100 Hm. *p < 0.05, **p < 0.001 vs. Ctrl, n = 3.

SUPPLEMENTARY EXPERIMENTAL METHODS

Carbon Nanotubes

MWCNTs used in this study were obtained from Hodogaya Chemical Company (MWNT-7, lot #061220-31). They were manufactured using a floating reactant catalytic chemical vapor deposition method followed by high thermal treatment in argon at 2,500 °C furnace. They were characterized by electron microscopy, X-ray photoelectron spectroscopy, Raman spectroscopy, and by dynamic light scattering (see Results and Discussion section)

Animals

Male C57BL/6 J mice (7 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME) and allowed to acclimate for one week before use. All animals used in this study were housed in an AAALAC accredited, specific pathogen-free, environmentally controlled facility. All animal procedures were approved by the NIOSH Institutional Animal Care and Use Committee. Mice were maintained on Harlan Teklad Rodent Diet 7913 (Indianapolis, IN) and tap water was provided *ad libitum*.

Exposure of Mice to MWCNT

Suspensions of MWCNT were prepared in dispersion medium (DM; Ca^{2+} and Mg^{2+}-free phosphate buffered saline, pH 7.4, supplemented with 5.5 mM D-glucose, 0.6 mg/mL mouse serum albumin, and 0.01 mg/mL 1,2-dipalmitoyl-sn-glycero-3-phosphocholine). DM was developed and validated by our laboratory as a vehicle for nanotoxicology studies[28]. After the initial suspension in DM, the sample was put on ice and briefly sonicated (Branson Sonifier 450, 10W output, 50% duty cycle, 5 minutes) before use. Mice were exposed by oropharyngeal aspiration to either DM or 40 µg MWCNT. For oropharyngeal aspiration, mice were anesthetized with isoflurane (Abbott Laboratories, North Chicago, IL) and when fully anesthetized, the mouse was positioned with its back against a slant board and suspended by the incisor teeth using a rubber band. The mouth was opened, and the tongue gently pulled aside from the oral cavity. A 50 µL aliquot of sample was pipetted at the base of the tongue, and the tongue was restrained until at least 2 deep breaths were completed (but for not longer than 15 seconds). Following release of the tongue, the mouse was gently lifted off the board, placed on its left side, and monitored for recovery from anesthesia.

Tissue Processing

At the end of the experiments, mice were euthanized and the lungs were fixed by intratracheal perfusion with 1 mL of 10% neutral buffered formalin via trachea cannula. After fixation, the lungs were sliced into 2-3 mm thick tissue blocks and embedded in paraffin. Sections (5 µm

thick) were then cut and stained with Mayer's hematoxylin (Sigma Aldrich, St. Louis, MO) and trichrome (Sigma Aldrich) or Sirius Red (American Mastertech Scientific, Inc., Lodi, CA). The tissue sections were then dehydrated, mounted and visualized on a microscope.

Cell Culture

Normal human lung fibroblasts (NHLFs) were purchased from ATCC (Manassas, VA) and were cultured in fibroblast cell culture medium (FBM) supplemented with SingleQuot growth factors and 2% FBS (Lonza Walkersville, Inc., Walkersville, MD). NHLFs from passages 2-5 were used in this study and were maintained under a sub-confluence condition in FBM in an incubator at 37° C with 5% CO_2.

MWCNT Preparation for *in vitro* Studies

For cell culture studies, MWCNTs were prepared as 1 mg/mL suspension in water by sonication for 30 min (Hielscher ultrasonic processor, UIS259L; Ringwood, NJ) at amplitude 100% and cycle 1. Following the sonication, the suspension was additionally sonicated for 30sec×3 at 5 W output and 30% duty cycle in 1 min increments (Branson Sonifier 450; Danbury, CT). The suspension (1 mg/mL) of MWCNTs was then diluted into 0.1 mg/mL of 0.5% bovine serum albumin (BSA) PBS solution as a stock and kept at 4 °C. The MWCNT stock suspension was directly sonicated for 10sec ×3 at 5 W output and 30% duty cycle prior to use.

Transmission Electron Microscopy (TEM)

MWCNTs were diluted in double distilled water (ddH_2O) followed by vortexing, and a drop of the suspension was placed on a formvar-coated copper grid and allowed to air dry. Images were photographed under a JEOL 1220 transmission electron microscope (Peabody, MA). The particles were dispersed in ddH_2O and filtered with a 0.2 mm Nucleopore filter. The filter was attached with double-stick carbon tape on an aluminum mount and sputter coated with gold/palladium. Images were collected on a Hitachi (Tokyo, Japan) S-4800 field emission scanning electron microscope.

X-ray Photoelectron Spectroscopy (XPS)

XPS analysis was carried out with a Physical Electronics Versa Probe 5000 XPS (Chanhassen, MN). MWCNT powder was pressed into a small pellet and evacuated in an entry chamber for approximately 2 h before being transferred to an ultra-high vacuum chamber for analysis. The sample was stimulated by a focused aluminum K-a X-ray source at 1486 eV energy and 25 W power with an X-ray spot size of 100 mm. A survey scan was carried out using an analyzer pass energy of 117.40 eV, and high-resolution scans for carbon.

Raman Spectroscopy

Raman spectra of MWCNTs were collected using a Renshaw Invia Raman spectrometer with a CCD detector (Hoffman Estates, IL). MWCNT powder was directly placed on a microscope glass slide, which was then mounted under the 50x objective lens of the Raman microscope. Samples were excited with a 532 nm green laser at around 0.23 mW laser power for data collection.

Zeta Potential Measurements

The electrophoretic mobility of particles was determined by light scattering in an applied electric field and converted to values of Zeta potential using Henry equation. All measurements were performed at 25° C using a Malvern Zetasizer Nano ZS90 (Worcestershire, UK) equipped with a 633 nm laser at a 90° scattering angle. Samples were equilibrated inside the instrument for 2 min, and five measurements (10 sec delay between measurements) each consisting of five runs (2 sec delay between runs) were recorded.

Cell Proliferation

Cells were seeded at the density of 1.5×10^4 cells per well in 96-well plates (Fisher, Waltham, MA, USA). After a 12 h incubation period, the cells were treated with different concentrations of MWCNTs for 1, 3 and 5 days. After the treatment, 20 µL of MTS CellTiter-96 aqueous one solution (Promega, Madison, WI, USA) were added to each well and the cells were incubated at 37 °C for 3 h. Viable cells cleaved the reagent's tetrazolium salt to a soluble formazan dye, resulting in a color change proportional to the number of live cells. Absorbance was measured

at 490 nm with a reference wavelength at 630 nm using a BioTek plate reader (BioTek, Winooski, VT, USA).

Focus Formation and Sphere Formation Assays

For focus formation assay, cells were seeded at the density of 2.5×10^3 cells per well in 6-well plates and allowed to grow for 10-14 days in the presence or absence of MWCNTs. At the end of the experiments, cells were fixed, stained with crystal violet, and examined under a light microscope. For sphere formation assay, cells were grown under non-adherent and serum-free culture conditions as previously described as selective for stem cells.[25,29] Briefly, 4×10^3 cells were suspended in 0.8% methylcellulose-based serum-free medium (Stem Cell Technologies, Vancouver, Canada) supplemented with 20 ng/mL epidermal growth factor (BD Biosciences, San Jose, CA), 10 ng/mL basic fibroblast growth factor, 5 µg/mL insulin (Sigma-Aldrich, St. Louis, MO), and 0.4% BSA (Sigma-Aldrich) in ultra-low adherent 6-well plates. The cells were cultured for two weeks and tumor spheres were examined under a light microscope. The number of spheres exceeding 20 µm in diameter was quantified. All experiments were performed in triplicates and repeated at least twice, with 5 fields of view analyzed for each replicate.

ALDH Activity and Cell Sorting

ALDH activity was determined by flow cytometry using an Aldefluor assay kit (Stem Cell Technologies), according to the manufacturer's instructions. Briefly, for each sample, $1x10^6$ cells were incubated in the Aldefluor assay buffer with activated Aldefluor substrate for 45 min at 37 ºC in the presence or absence of a specific ALDH inhibitor, diethylaminobenzaldehyde (DEAB). Tubes treated with both Aldefluor substrate and DEAB served as a negative control for each sample. ALDH converts Aldefluor substrate into a fluorescent product, and cells with high ALDH activity ($ALDH^{high}$) were detected by the BD Fortessa cell analyzer (BD Biosciences, San Jose, CA). The flow cytometry gates were set to obtain 0.01% $ALDH^{high}$ cells in the substrate + inhibitor tubes for each sample. Cells with high and low ALDH activity were sorted using

FACSAria fluorescence activated cell sorter (BD Biosciences). Sorting gates for FACS were drawn relative to fluorescence intensity of the cells. Sorted cells were captured in cold media (4°C) and immediately plated in chamber slide at 37 °C with 5% CO_2.

Immunoblotting

After specific treatments, cells were washed with PBS and lysed on ice with modified RIPA buffer containing protease and phosphatase inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN) for 30 min. The cell lysates were sonicated briefly and centrifuged at 14,000×g for 20 min. Lysate proteins (40 μg) were resolved under denaturing conditions by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked for 1 h in 5% nonfat dry milk in TBST (25 mM Tris-HCl, pH 7.4, 125 mM NaCl, 0.05% Tween 20) and incubated with primary antibodies at 4 °C overnight with gentle shaking. Membranes were rinsed three times with TBST for 10 min each followed by incubation with horseradish peroxidase-conjugated second antibodies for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence detection reagents (Millipore Corporation, Billerica, MA). β-Actin was blotted to ensure equal loading of the samples and data were quantified using image J densitometry software.

Immunostaining

Mouse lung sections in paraffin were deparaffinized and rehydrated. Antigens were retrieved with 10 mM sodium citrate solution in the microwave for 20 min. Slides were blocked with 3% BSA/0.1% Tween 20 in 1× PBS blocking buffer for 1 h and then incubated with anti-ALDH1A1 antibody (1:100 dilution, Abcam, Cambridge, MA), ABCG2 (1:50 dilution, Sigma Aldrich, St. Louis, MO). After rinsing with PBS three times, the slides were incubated with Alexia 549 secondary antibodies (Invitrogen, Carlsbad, CA) for 1 h or CD90/Alexia488 (1:100 dilution, Biolegend, San Diego, CA), αSMA/alexia 488 (1:100 dilution, Abcam, Cambridge, MA) for 30 min, followed by adding a mounting solution with DAPI (Vector Laboratories Inc, Burlingame,

CA). Images were taken using a Carl Zeiss fluorescence microscope with a Zen lite digital imaging software (Jena, Germany). For quantitation of staining, the percentage of positive cell staining was calculated per mm^2 using an image analysis software.

Statistical Analyses

Results are expressed as means ± s.d. All values were derived from at least three independent experiments. Differences between groups were evaluated by one-way analysis of variance (ANOVA) followed by the Student's *t* test. For all analyses, two-sided *p* values of ≤ 0.05 were considered statistically significant.