

Supplementary Information

Supplementary Information includes Supplementary Figure S1, Supplementary Table S1, Supplementary Video S1 and S2 and Supplementary Materials and Methods.

Supplementary Figure

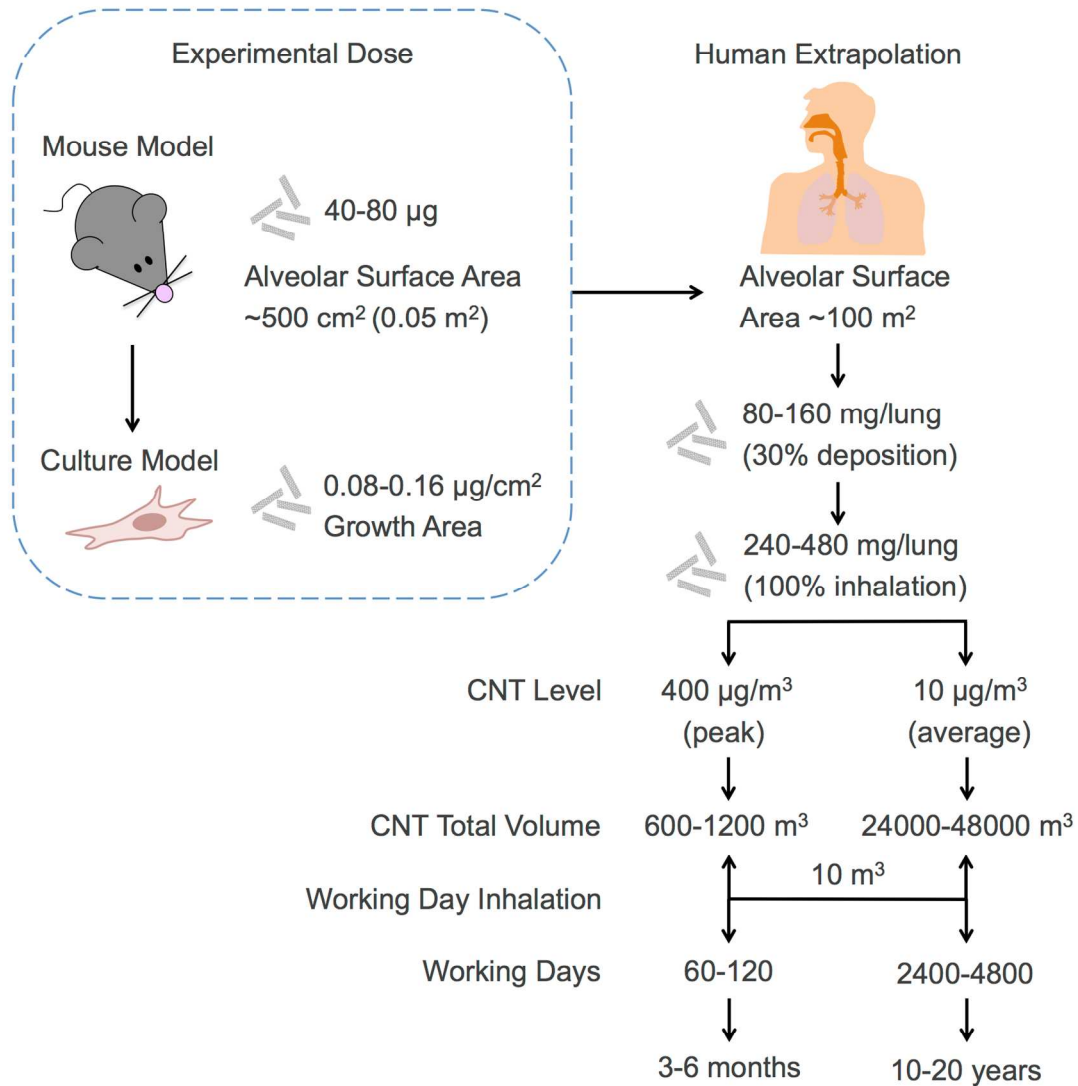


Figure S1. Extrapolation of the carbon nanotube experimental dose in mouse and cell culture models to human exposure scenarios in the workplace.

Supplementary Table

Table S1. *In vitro* dosimetry of carbon nanotubes.

Plate Format	Growth Area (cm ²)	Cell Density	Medium Volume (mL)	CNT Surface Dose 0.08-0.15 µg/cm ²		
				Concentration (µg/mL)	CNT Mass (µg)	Mass/Cell (pg/cell)
24 well ^a	1.9	3 × 10 ⁴	0.400	0.380-0.712	0.152-0.285	5.000-9.500
6 well ^b	9.5	1.5 × 10 ⁵	2.000	0.380-0.712	0.760-1.425	5.000-9.500

^a24 well plate format was for cytotoxicity and cell proliferation, Sircol soluble collagen, fibroblastic nodule and immunofluorescence assays; ^b6 well plate format was for Western blot and side population analysis.

Supplementary Video

Video S1. 3D reconstruction of Z-stack confocal image series of fibroblastic nodules induced by SWCNT.

Video S2. 3D reconstruction of Z-stack confocal image series of fibroblastic nodules induced by MWCNT.

Supplementary Materials and Methods

CNT Characterization and Preparation

SWCNT were obtained from Carbon Nanotechnology (CNI, Houston, Texas) and were purified by acid treatment to remove metal contaminants, while MWCNT were obtained from Mitsui & Company (New York, NY). Elemental analysis was performed by nitric acid dissolution and ICP-AES (NMAM #7300). The specific surface area was measured at -196 °C by the nitrogen absorption-desorption technique (BET method)

using a SA3100 Surface Area and Pore Size Analyzer (Beckman Coulter, Fullerton, CA). The diameter and length distribution of the SWCNT were measured by field emission scanning electron microscopy. SWCNT and MWCNT were dispersed using bovine serum albumin (BSA). Briefly, 1 mg of the CNT were dispersed in 1 mL of phosphate-buffer saline (PBS) containing 5 mg of BSA using light sonication and were diluted in culture medium to obtain the desired concentrations.

Cells and Reagents

Primary normal human lung fibroblasts were obtained from Lonza (Allendale, NJ) and were cultured in Clonetics[®] Fibroblast Growth Medium-2 (Lonza) which contains 2% fetal bovine serum (FBS), 0.1% insulin, 0.1% basic fibroblast growth factor (rhFGF-B) and 0.1% gentamicin. The cells were maintained in a humidified atmosphere of 5% CO₂ at 37 °C. Human-derived TGF- β was obtained from R&D Systems (Minneapolis, MN). Poly-L-lysine and Hoechst 33342 were obtained from Sigma-Aldrich (St. Louis, MO). Antibody for collagen type I was from Fitzgerald (Acton, MA). Antibodies for α -SMA and ALDH1A1 were from Abcam (Cambridge, MA). Antibody for ABCG2 (BRCP1) was from Milipore (Billerica, MA), and horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA). DAPI and Alexa Fluor secondary antibodies were obtained from Molecular Probes (Grand Island, NY).

Human Lung Specimens and Protein Lysates

Frozen and formalin-fixed, paraffin-embedded human lung specimens, and protein lysates of lung fibrotic and matched normal tissues were obtained from Origene (Rockville, MD). Patient and matched normal sample IDs of the lung specimens and

protein lysates are (1) #PA00001079 and #PA0000E89; (2) #FR0006697 and #FR0000668D; and (3) #FR0002FB8C and #FR00038A36.

Cytotoxicity and Cell Proliferation

Cytotoxicity and cell proliferation were determined by MTT colorimetric assay. After specific treatments, cells in 24-well plates were incubated with 500 µg/mL of MTT for 4 h at 37 °C. The intensity of the formazan product was measured at 550 nm using a microplate reader. Relative cell viability was calculated by dividing the absorbance of treated cells by that of the non-treated cells in each experiment.

Western Blot Analysis

After specific treatments, the cells were incubated in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 10% glycerol, 1 mM Na₃VO₄, 50 mM NaF, 100 mM phenylmethylsulfonyl fluoride, and a commercial protease inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN) at 4 °C for 20 min. Cell lysates were collected and determined for protein content using the Bradford method (Bio-Rad Laboratories, Hercules, CA). Proteins (50 µg) were resolved under denaturing conditions by 7.5-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Bio-Rad Laboratories). The transferred 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 the appropriate primary antibodies at 4 °C overnight. Membranes were washed twice with TBST for 10 min and incubated with HRP-coupled isotype-specific secondary antibodies for 1 h at room temperature. The immune complexes were detected by an enhanced chemiluminescence detection system and quantified using analyst/PC densitometry

software. β -actin was used as a loading control. The intensity of target bands was normalized to that of β -actin. Mean data from at least three independent experiments (one of which is shown) were then calculated by dividing the normalized intensity of each sample by that of control.

Sircol[®] Soluble Collagen Assay

Sircol[®] red collagen assay kit (Biocolor Ltd., UK) was used to measure soluble collagen, according to the manufacturer's protocol. Briefly, cells were cultured at the density of 3×10^4 cells/well in 24-well plates and treated with CNTs or TGF- β for 48 h. Cell supernatants were collected and mixed with Sircol[®] red dye reagent for 30 min. A 200 μ L aliquot of the mixed supernatant was transferred to a 96-well plate and analyzed for absorbance at 560 nm using a microplate reader (BioTek, Winooski, VT).

Growth and Counts of Fibroblastic Nodules

Cells at the density of 3×10^4 cells/well were seeded onto poly-L-lysine-coated glass coverslips in 24-well plates and allowed to adhere overnight. The cells were then deprived of serum for 8 h and then treated with SWCNT, MWCNT, or TGF- β for 16 h, which is the optimal time for fibroblastic nodule formation. For each culture, three cover slides were analyzed and counted (Leica Microsystems, Bannockburn, IL) by two independent observers blinded to the treatment.

Immunofluorescence

For immunofluorescence staining, cells were seeded on rat type I collagen-coated coverslips ($5 \mu\text{g}/\text{cm}^2$), fixed with 3.7% paraformaldehyde for 15 min, incubated in 50 mM glycine for 5 min, permeabilized and blocked with 0.5% saponin, 1.5% BSA, and

1.5% normal goat serum for 30 min. Coverslips were then incubated with the appropriate primary antibodies at 4 °C overnight followed by secondary Alexa Fluor 488-, 546-, or 647-conjugated antibodies and phalloidin (Molecular Probes) for 2 h. After washing, cells were mounted on a coverslip using ProLong[®] Gold antifade reagent with DAPI (Molecular Probes). Cells were visualized with a Zeiss LSM 510 confocal microscope on an AxioImager Z1 platform using a 40x objective lens (Carl Zeiss, Jena, Germany).

For frozen tissue staining, frozen tissue sections were fixed and permeabilized in -20 °C acetone for 10 min, and incubated in 1% BSA and 10% normal goat serum for 1 h. Slides were then similarly stained with the appropriate primary antibodies and secondary Alexa Fluor 647-conjugated antibodies.

Side Population Analysis and Isolation

Cells were detached by trypsinization and 1×10^6 cells were labeled with 5 µg/mL of Hoechst 33342 in DMEM-F12 medium containing 2% FBS in the presence or absence of 25 µM ABCG2 inhibitor fumitremorgin C (FTC; EMD Biosciences, San Diego, CA) at 37 °C for 90 min. The cells were then centrifuged and resuspended in ice-cold Hank's buffer salt solution (HBSS). SP analysis and sorting were performed using BD FACSAria fluorescence-activating (flow cytometry)-based cell sorter (BD Biosciences, San Jose, CA). The Hoechst dye was excited with a UV laser and its fluorescence was measured with both 450/20 filter (Hoechst Blue) and 675 LP filter (Hoechst Red). SP fraction was calculated based on the disappearance of SP cells in the presence of FTC using the formula: SP percentage in the absence of FTC – SP percentage in the presence of FTC.

Immunohistochemistry

Paraffin-embedded tissue sections were deparaffinized with xylene and a gradient ethanol series and immunolabeled with appropriate primary antibodies at 4 °C overnight followed by the secondary antibody of biotinylated horse anti-mouse IgG (Vector, Berlingame, CA) for 2 h. Immunoactivity was detected using Vectastain ABC reagent (Vector) with 3, 3'-diaminobenzidine as a substrate.

Statistical Analysis

The data represent means \pm SD from three or more independent experiments. Statistical analysis was performed by Student's *t* test at a significance level of $p < 0.05$.