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Carbon Nanotubes Induced Fibrogenesis on Nanostructured Substrates[†]

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Abstract

While the rapidly evolving nanotechnology has shown promise in electronics, energy, healthcare and many other fields, there is an increasing concern about the adverse health consequences of engineered nanomaterials. To accurately evaluate the toxicity of nanomaterials, *in vitro* models incorporated with *in vivo* microenvironment characteristics are desirable. This study aims to delineate the influence of nanotopography on fibrogenic response of normal human lung fibroblasts to multi-walled carbon nanotubes (MWCNTs). Nanoscale gratings and pillars of various heights were fabricated on polydimethylsiloxane substrates. Cell spreading and biomechanics were measured, and fibrogenic responses including proliferation, collagen production and reactive oxygen species generation of the fibroblasts grown on the nanostructured substrates in response to MWCNTs were assessed. It was observed that the cells could be largely stretched on shallow nanogratings, leading to stiffer cytoskeleton and nucleus, enhanced cell proliferation and collagen production, and consequently, toxic response sensitivity of the fibroblasts was undermined. In contrast, the cell spreading and stiffness could be reduced using tall, isotropic nanopillars, which significantly improved the cell toxic sensitivity to the MWCNTs. In addition to highlighting the significant influence of cell-nanotopography interactions on cell sensing CNTs, this study contributed to development of physiologically relevant *in vitro* models for nanotoxicology study.

Graphical abstract

[†]Electronic Supplementary Information (ESI) available: Atomic force microscopic measurement of cytoskeletal and nuclear stiffnesses, characterization of MWCNTs, expression of collagen I $\alpha 1$ and $\alpha 2$, and a table of primary antibodies used for western blot. See DOI: 10.1039/x0xx00000x

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The fibrogenic response of lung fibroblasts to carbon nanotubes is regulated by substrate nanotopography through modulating cell spreading and elongation.

Introduction

When the rapidly evolving nanotechnology improves the quality of human life through its impacts on electronics, energy, healthcare and many other fields, occupational and consumer exposure to engineered nanomaterials is expected to be accelerated.¹⁻³ For instance, the global market for carbon nanotubes (CNTs) is estimated to reach a trillion dollars in the next decade with their use affecting millions of workers and users.⁴ Metal oxide nanoparticles have also been increasingly used in polymer electrolyte fuel cells.⁵ Nanomaterials can come in contact with human organs, and especially be inhaled to target lung. The small size of nanomaterials permits their deposition to deep lung tissues including the interstitium with very low clearance. Their bio-persistence enhances long term exposure adverse effects thus resulting in significant public health problems.^{6, 7} It has been known that CNTs can produce diverse biological and toxicological effects, including inflammation, tissue damage, and lung fibrosis, a fatal and incurable disease with no known effective treatment.⁸⁻¹¹ However, the adverse health effects of other nanomaterials are largely unknown. To establish the safety regulation of nanomaterials handling and applications and to develop proper personal protective equipment, it is highly desirable to determine unintended toxic effects of nanomaterials.¹²

Both *in vivo* and *in vitro* models are being used to evaluate nanomaterials for their toxicity. Animal studies are necessary but costly, time-consuming and facility limited. The use of *in vitro* models can provide a rapid and inexpensive way to assess the potential toxicity of nanomaterials. They can also facilitate mechanistic investigations of toxicological pathways and signaling molecules at cellular and molecular levels.¹³ However, the majority of current *in vitro* models lack characteristics of *in vivo* microenvironment, leading to the cell behavior deviated from *in vivo* phenotypes and responsiveness.¹⁴ For instance, while some *in vitro* studies showed minimal toxicity for cerium oxide (CeO₂) nanoparticles,^{15, 16} animal studies showed that CeO₂ induced lung inflammation and fibrosis *in vivo*.¹⁷⁻¹⁹ Therefore, it is critical to incorporate the microenvironmental characteristics into development of physiologically relevant *in vitro* models, which can provide reliable, rapid and inexpensive methods for toxicology studies and risk assessment of nanomaterials.

In most living tissues, cells typically reside in and interact with extracellular matrix (ECM). The ECM is constructed from diverse biomacromolecules with size ranging from several to

hundreds of nanometers²⁰ and often includes significant nanoscale topography.²¹⁻²³ It has been shown that inhaled CNTs rapidly enter the interstitium to stimulate collagen production and induce progressive interstitial lung fibrosis in weeks in mice.^{9, 24} The interstitial matrix displays an interrelated framework of nanoscale fibrous proteins such as collagen and elastin.^{8, 25} Nanoscale structures have been shown to have pronounced influence on cell behavior, from cell adhesion and spreading to proliferation and differentiation.²⁶⁻²⁸ For instance, our and other labs observed that human fibroblasts responded differently to nanoscale topography.²⁹⁻³¹ Fibroblasts displayed a highly spread morphology containing well-defined stress fibers on shallow nanotopographies, but had a reduced cell spreading morphology with diffuse actin and fewer stress fibers when the nanotopography height increased.^{29, 30} The height of nanotopography was found to be an effective regulator of nuclear deformation, through which cell proliferation and collagen I production of fibroblasts could be modulated.³¹

In this study, we investigated the nanotopographical effects on fibrogenic response of normal human lung fibroblasts (NHLFs) to multi-walled CNTs (MWCNTs). Representative nanotopographies (nanoscale gratings and pillars of different heights) were engineered on polydimethylsiloxane (PDMS) substrates. Cell spreading and cell biomechanics of the fibroblasts on nanotopographies were measured. Nanotopographical effects on the fibrogenic response of the fibroblasts to MWCNTs were assessed via cell proliferation, collagen I and oxidative stress generation as potential fibrosis biomarkers. Our observations suggested that nanotopography had a pronounced influence on fibroblasts response to the CNTs.

Experimental

Fabrication of nanotopography

Nanotopographies were fabricated by employing electron beam lithography (EBL), transferred to PDMS substrates by using soft lithography and then expanded to a large area by applying a stitch technique, which was previously reported.³¹ Briefly, the lateral and vertical dimensions of nanotopography were defined by EBL and reaction ion etching processes, respectively. When a mixture of PDMS resin and curing agent (Sylgard 184 kit; Dow Corning, MI, USA) was cast on the EBL-written mold, a slightly higher concentration of curing agent, i.e., a 10:1.05 w/w ratio of PDMS resin/curing agent, was applied to reduce the amount of uncured oligomers.³² Multiple PDMS replicates were stitched by aligning the nanopatterned surfaces on a silicon wafer and gluing the backside with a PDMS adhesive layer.³³ The stitched mold was then nanoimprinted into polystyrene (PS) substrates, which served as a master mold to produce working PDMS nanotopographies.

Prior to cell seeding, the PDMS substrates including nanotopographies and flat surfaces were sterilized by using 70% ethanol and then UV exposure, each for 30 min, followed by incubating with 20 µg/mL fibronectin (human; Corning, New York, NY, USA) for 30 min.

Preparation, dispersion and characterization of MWCNTs

MWCNTs (Mitsui & Company, Japan) was dispersed as previously described.³⁴ Briefly, 1 mg of MWCNTs was dispersed in 1 mL of phosphate buffered saline (PBS; Life Technologies, Carlsbad, CA, USA) containing 5 mg of bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) using light sonication in a biological safety hood. The MWCNTs stock solution was stored at 4°C and further diluted in the culture medium to obtain the desired concentrations (0.06 – 0.6 µg/cm²) before use.

The size of MWCNTs was measured by using atomic force microscopy (AFM). The sample was prepared by spraying the diluted MWCNTs on a silicon substrate, followed by air dry. SEM imaging was performed in a field emission SEM (JEOL JSM-7600F, Peabody, MA, USA). AFM measurement was performed using a Veeco Nanoscope Multi Mode scanning probe microscope in the tapping mode under ambient conditions. Topography images were recorded at the fundamental resonance frequency of the cantilever, with a typical scan rate of 1 Hz and a resolution of 512 samples per line.

Cell culture

NHLFs (CRL1490 cells; ATCC, Manassas, VA, USA) were cultured in fibroblast basal medium supplemented with 2 % fetal bovine serum (FBS), 0.1% recombinant human fibroblast growth factor basic (rhFGF-B), 0.1% insulin, and 0.1% gentamicin/amphotecin-B (Lonza, Walkersville, MD, USA), and used in passages 3 – 6.

SEM observations

The PDMS substrates with cells grown were washed with PBS and fixed in a mixture of 4% paraformaldehyde (PFA; Electron Microscopy Sciences, Hatfield, PA, USA) and 2% glutaraldehyde (Fisher Scientific, Pittsburgh, PA, USA) in PBS at ambient for 4 h. The samples were then rinsed with deionized water and progressively dehydrated using a Tousimis Autosamdri-815 CO₂ Critical Point Dryer (Tousimis Co., Rockville, MD, USA).

The PDMS substrates with and without cells grown were sputter-coated with a gold layer of ~10 nm thick by using a Denton Vacuum Desk V sputter unit (Denton Vacuum, LLC, Moorestown, NJ, USA), and imaged in a JEOL JSM-7600F SEM.

Cytoskeletal and nuclear stiffness measurement

The stiffness of cell cytoskeleton and nucleus were measured using an Asylum MFP3D-BIO AFM (Asylum Research, Goleta, CA, USA) integrated with a Nikon Ti-S inverted fluorescence microscope (Melville, NY, USA) (see Fig. S1 in ESI).

Before AFM measurements, cells were live-stained with 2 µg/mL Hoechst (Life Technologies) in culture media for 30 min, rinsed with 37°C Dulbecco's phosphate-buffered saline (DPBS; Sigma-Aldrich), and then supplied again with culture media.

The nucleus was identified under the fluorescence microscope and positioned underneath the AFM cantilever. Three measurements were taken at three different positions above the nucleus of each cell. Starting from a few micrometers above the cell, the AFM cantilever

(Bruker, Billerica, MA, USA), which had a nominal spring constant of 0.06 N/m and was calibrated using a thermal-tune method,³⁵ was lowered to indent into the cytoplasmic membrane (Fig. S1A) and then into the nucleus (Fig. S1B). When the cantilever was lowered and the indentation force reached a preset value of 15 nN, a sinusoidal signal with an amplitude of 25 nm and a frequency of 10 Hz was added to the cantilever position for 1.5 sec (Fig. S1C). Fig. S1D showed a representative force – indentation curve recorded from the AFM indentation process. The resistance from the cytoskeleton above the nucleus caused the initial increase in indentation force, resulting in the segment from Point A to Point B. The stiffness value obtained by fitting the first 400 nm of this segment to the Hertz model^{36, 37} was representative of the stiffness of the cytoskeletal layer on top of the nucleus. The total indentation distance from Point A to Point B approximately described the thickness of the cytoskeletal layer. Beyond Point B, a significant increase in steepness of the force – indentation curve was observed. There was also a detected phase lag between the sinusoidal force and indentation (Fig. S1E), indicating the viscoelastic property of nucleus. The elastic modulus of nucleus was calculated from the relationship between the sinusoidal force and indentation.^{38, 39}

Cell proliferation

NHLFs were seeded on the PDMS substrates at the density of 7,500 cells/cm² and cultured overnight to allow cells to adhere. 0.2 µg/cm² MWCNTs were added and incubated with the cells for 48 h. Cell proliferation was detected by using CellTiter 96[®] Aqueous One Solution Cell Proliferation kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Briefly, the medium in each well was replaced with 100 µL fresh culture medium containing 20 µL of CellTiter 96[®] Aqueous One Solution Reagent. The 96-well plates were incubated for additional 4 h at 37°C. The absorbance of each well at a wavelength of 490 nm was measured by a FLUOstar OPTIMA Microplate Reader (BMG LABTECH, Cary, NC, USA). Culture medium containing reagent was set as background.

Immunofluorescence staining

NHLFs on the PDMS substrates were fixed with 4% PFA for 30 min at room temperature, permeabilized and blocked in PBS solution containing 0.03 g/mL BSA, 1% goat serum (Sigma-Aldrich), and 0.2% Triton-X 100 for 1 h. Primary antibody conjugation was performed in PBS solution with 0.2% Triton-X 100 for 2h, followed by secondary antibody labelling for 1 h, both at room temperature. Type I collagen was stained with anti-collagen rabbit polyclonal antibody (1: 200; Fitzgerald, Concord, MA, USA), followed by the Alexa Fluor 488 goat anti-rabbit secondary antibody (1:200; Life Technologies). F-actin was stained with Alexa Fluor 488 phalloidin (Life Technologies), and the nuclei were counterstained and mounted using ProLong Gold Antifade Reagent with 4,6-diamidino-2-phenylindole (DAPI; Life Technologies).

Images were taken by using a Nikon Ti Eclipse fluorescent microscope. Cell area and roundness were analyzed by using ImageJ (<http://imagej.nih.gov/ij/>).

Reactive oxygen species (ROS) staining

NHLFs were seeded on the PDMS substrates at a density of 7,500 cells/cm². After overnight culture, the cells were treated with 0.2 µg/cm² MWCNTs and incubated for another 16 h. Cells cultured on nanotopographies without any treatment were set as control. The culture medium was then replaced with 1 mL Hank's balanced salt solution (HBSS) containing 10 µM dichlorodihydrofluorescein diacetate (DCF-DA) and incubated for additional 30 min. Cells were finally fixed in 4% PFA solution and the nuclei were visualized using ProLong Gold Antifade Reagent with DAPI. At least 10 images for each group were taken under Nikon Ti Eclipse fluorescent microscope with the same setting. The fluorescent density of images was analyzed by imageJ and the ROS production was calculated by using the following formulation.

$$\frac{\text{ROS production}}{\text{Cell}} = \frac{\text{Image fluorescence intensity} - \text{background fluorescence intensity}}{\text{Cell number}}$$

Western blot

The whole cell protein was extracted by lysing cells with radioimmune precipitation assay (RIPA) buffer (Santa Cruz Biotechnology, CA, USA) containing protease and phosphatase inhibitors for 30 min on ice, separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Darmstadt, Germany). The PVDF membranes were then blocked with 5% nonfat milk in 1× Tris-buffered saline with 0.1% Tween-20 (TBST), followed by blotting with primary antibodies (see Table S1 in ESI) at 4°C overnight with shaking, and incubating with a horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich) for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence detection reagents (EMD Millipore) and acquired by C DiGit Blot Scanner (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis

Cell area (n = 50), roundness (n = 40), cell stiffness (n > 10), proliferation (n = 3), collagen I expression (n = 3) and ROS production (n > 10) of NHLFs were measured from three independent replicates for each condition and presented as mean ± standard error of mean (S.E.M.). Student's t-test was used to determine the statistical difference. Statistically significant differences were considered at a level of $p < 0.05$.

Results and Discussion

Characterization of nanotopographies

Previously, we systematically investigated the influence of geometry, feature size and height of nanotopography on cell adhesion, nuclear volume and cell function of NHLFs.³¹ It was observed that the nuclear volume of the fibroblasts on the nanogratings (500 nm in line width, one time line width in spacing and 150 nm in height; labelled as 500-1-150) and nanopillars (500 nm in diameter, 1.9× diameter in center-to-center spacing and 150 nm in height; labelled as 500-1.9-150) was significantly larger than those on flat control surfaces,

but became significantly smaller than the flat controls when the nanotopography height increased to 560 nm (i.e., on 500-1-560 and 500-1.9-560). The alteration in nuclear volume modulated by nanotopography resulted in changes in cell function such as cell proliferation, exogenous gene transfection and ECM remodelling. Moreover, isotropic nanopillars and anisotropic nanogratings represent two major scenarios in terms of length and ordering of nanofeatures, and the structure of fibrous ECM proteins observed *in vivo* lies between these scenarios.⁸ Therefore, the current study examined the influence of the aforementioned four typical nanotopographies on fibrogenic responses, i.e., cell proliferation, collagen I production and reactive oxygen species (ROS) generation of the fibroblasts to MWCNTs, and explored the underlying mechanism of nanotopography regulated fibroblasts sensing CNTs. The SEM images of these nanotopographies and the flat control surface were displayed in Fig. 1, and their heights were confirmed by AFM measurement.³¹ This design of nanotopographies allow us to investigate the influence of geometry and dimensions of nanotopography on fibrogenic response of the fibroblasts to the CNTs.

Cell spreading on nanotopographies

The fibroblasts displayed different morphology on the substrates. The cells spread randomly with multipolar morphology on the flat control surfaces and nanopillars, but elongated along the nanograting direction, in particular largely aligned along the nanograting direction of 500-1-560 (Fig. 2A). The cell elongation was quantified by measuring cell roundness, which was defined as $4 \times [\text{cell area}] / (\pi \times [\text{major axis}]^2)$. Cell roundness was the inverse of cell aspect ratio; a smaller roundness value indicates a larger cell elongation. No significant difference in cell roundness between the nanopillars and the flat controls was observed. However, the cell roundness on the nanogratings was significantly smaller than the flat controls (Fig. 2B), being in line with the elongated cell morphology. In addition, the cell roundness on 500-1-560, 0.12 ± 0.01 was significantly smaller than that on 500-1-150, 0.22 ± 0.02 ($p = 0.003$), indicating that the cells were further elongated or stretched on the deeper nanogratings. The height of nanotopography also showed a pronounced influence on cell area. The cell areas on the nanotopographies of 150 nm height were similar to the flat controls. However, the average cell areas on the nanotopographies of 560 nm height, $2404.7 \pm 139.4 \mu\text{m}^2$ on 500-1.9-560 and $2081.9 \pm 113.3 \mu\text{m}^2$ on 500-1-560 were significantly smaller than their counterparts of 150 nm height, $3042.5 \pm 176.7 \mu\text{m}^2$ on 500-1.9-150 and $2773.4 \pm 199.3 \mu\text{m}^2$ on 500-1-150, respectively (Fig. 2C). The observations suggested that the cell spreading (cell elongation and area) was profoundly affected by the geometry and dimensions (i.e., height in this study) of underneath nanotopography.

SEM observations provided further information on the cell-nanotopography interactions (Fig. 3). On the nanotopographies of 150 nm height, the cell could reach the top and bottom of the nanotopography and completely confine on the substrate surface. By comparison, on the deeper nanotopographies (500-1.9-560 and 500-1-560) the lamellipodia of the fibroblasts stayed on the top surface of the nanotopographies but could not bend to reach the bottom, and the filopodia extended on the top surface of nanotopographies, leading to a reduced cell area. Evidently, the differential cell-nanotopography contact area/adhesion resulted in difference in cell spreading.

At the nanoscale, the elasticity of cytoplasmic membrane may prevent a radical bending.⁴⁰ Our observations agreed with previous reports on the effects of feature size⁴¹ and height⁴² on cell–nanotopography adhesion. When the nanotopography was tall or the spacing was small, the cytoplasmic membrane could bridge over the top of nanotopography rather than reaching the bottom, accompanied with reduced cell area and nuclear volume. When the nanotopography was shallow, the cytoplasmic membrane could confine over the substrate surface regardless of the contour of nanotopography, accompanied with enlarged cell spreading area and nuclear volume. The geometry of nanotopography, on the other hand, affected cell elongation. Nanogratings provided continuous contact guidance to elongate the cells while isotropic, discrete nanopillars favored random orientation of the cells.

Cytoskeletal and nuclear stiffness of NHLFs on nanotopographies

Cell spreading was expected to affect cell biomechanics, and thus we measured the cytoskeletal and nuclear stiffness of the fibroblasts on the nanotopographies as summarized in Fig. 4. When the cell spread on the substrate, the cytoskeleton at the cell periphery could be very thin and the measured stiffness was contributed from the mechanical properties of both the cytoskeleton and the underneath substrate. Therefore, we measured the stiffness of cytoskeletal layer above the nucleus (see Figure S1 in ESI). The height of nanotopographies showed a significant influence on cytoskeletal stiffness (Fig. 4A). The cytoskeleton of the fibroblasts grown on the nanotopography of 150 nm height was significantly stiffer than its counter part of 560 nm height, i.e., 4.61 ± 0.71 kPa on 500-1.9-150 vs. 1.80 ± 0.38 kPa on 500-1.9-560 ($p = 0.006$), and 5.70 ± 0.48 kPa on 500-1-150 vs. 3.66 ± 0.79 kPa on 500-1-560 ($p = 0.04$). The nanogratings also increased the cytoskeletal stiffness compared with the nanopillars of same feature size and height and similar spacing (500 nm for the nanogratings and 450 nm for the nanopillars) although the difference was not statistically significant. For instance, the cytoskeletal stiffness on 500-1-560 was 3.66 ± 0.79 kPa higher than 1.80 ± 0.38 kPa on 500-1.9-560 ($p = 0.10$).

The height of nanotopographies had similar but less significant influence on nuclear stiffness compared with cytoskeletal stiffness. Shown in Fig. 4B, there was no significant difference in nuclear stiffness between the nanotopographies of 150 nm height and the flat controls. However, when the nanotopography height increased from 150 nm to 560 nm, the nuclear stiffness decreased. For instance, the nuclear stiffness decreased from 9.61 ± 1.11 kPa to 6.14 ± 1.15 kPa on the nanogratings ($p = 0.008$), and from 8.91 ± 0.87 kPa to 7.28 ± 0.92 kPa on the nanopillars ($p = 0.26$). The effects of nanotopography geometry on nuclear stiffness became insignificant. The results showed that the nuclear stiffness was less sensitive to underneath nanotopography than the cytoskeletal stiffness.

The nanotopography modulated cell biomechanics correlated well with the cell spreading. When the nanotopography height decreased, the cell perceived more substrate surface and spread more, accompanied with the increase in cell and nuclear size as well as stress fibers formation.^{31, 32} Our measurement also showed that the cytoskeletal layer between plasma membrane and nucleus became thinner when the cells spread more on shallow nanotopographies. For instance, the thicknesses of cytoskeletal layer were 0.96 ± 0.14 μ m on 500-1.9-150 and 1.39 ± 0.11 μ m on 500-1-150, thinner than 1.78 ± 0.16 μ m on 500-1.9-560

and $1.69 \pm 0.22 \mu\text{m}$ on 500-1-560, respectively. Cell spreading led to the increase in the cytoskeletal and nuclear stiffness (Fig. 4). Because the nucleus was stiffer than the cytoskeletal layer, which was implied by a significant increase in steepness of the curve after Point B in the force-indentation curve (see Fig. S1D of the ESI), the nanotopography had a more profound influence on cytoskeleton than nucleus. Nuclear stiffness was thus less sensitive to the underneath nanotopography than the cytoskeleton. Compared with isotropic nanopillars, the nanogratings elongated the cells, which might further increase their stiffness via an increase in actomyosin contractility.⁴³

CNT characterization and dose determination

The MWCNTs were purified by acid treatment to remove metal contaminants. Elemental analysis by nitric acid dissolution and inductive coupled plasma-atomic emission spectrometry (ICP-AES) showed that purified MWCNTs contained 99% elemental carbon and less than 1% of contaminants.⁴⁴ The length of the MWCNTs was measured to be in the range of several micrometers by using SEM, and the diameter was measured to be around 12 nm as indicated in the AFM profile (Fig. S2 in ESI).

The *in vitro* CNT treatment dose was determined based on *in vivo* CNT exposure data normalized to alveolar surface area in mice. Previously, the *in vitro* CNTs surface area dose of $0.08 - 0.16 \mu\text{g}/\text{cm}^2$ was used, relevant to *in vivo* exposure doses of $40 - 80 \mu\text{g}/\text{mouse}$.⁴⁴ In this study the doses of $0 - 0.6 \mu\text{g}/\text{cm}^2$ were examined, which covered the aforementioned range. Production of collagen I was assessed by western blot analysis after incubating the fibroblasts with MWCNTs at different doses for 48 h on flat PDMS surfaces. Collagen I expression monotonically increased with the increase in MWCNTs dose in the studied range (Fig. 5), which agreed with previous study.⁴⁵ Previous studies showed that MWCNTs at high doses ($> 0.6 \mu\text{g}/\text{cm}^2$) caused severe cytotoxicity.^{46, 47} The dose of $0.2 \mu\text{g}/\text{cm}^2$ was thus chosen for assessment of the nanotopography mediated fibrogenic response of the fibroblasts to MWCNTs.

Fibroblast proliferation

Since fibroblast proliferation and increased production of collagen matrix were hallmarks of lung fibrosis,¹⁰ we first evaluated the influence of nanotopography on cell proliferation with MWCNTs treatment. As shown in Fig. 6, in the absence of MWCNTs, the fibroblasts on the nanotopographies of 150 nm height (500-1.9-150 and 500-1-150) showed enhanced cell proliferation compared to their counterparts of 560 nm height (500-1.9-560 and 500-1-560). At the same height, the cell proliferation on the nanogratings was higher than that on the nanopillars although the nanogratings and nanopillars had same feature size, similar spacing, and same amount of increase in the surface area (30% and 112% increase compared to the flat surface for the heights of 150 and 560 nm, respectively). Different from the observations, previously we did not detect the difference in cell proliferation between the nanogratings and nanopillars at the same height,³¹ which was likely to be caused by a longer cell culture period, 48 h in this study than the previous 24 h. Our previous study showed the height of nanotopography as an effective modulator of cell behavior. On the nanotopographies of 150 nm height, the cells were largely spread out, accompanied with increased nuclear volume.³¹ Induced nuclear swelling was suggested to promote DNA

synthesis and thus enhance cell proliferation.⁴⁸ The differential cell proliferation on the nanotopographies of different geometry but same height could not be explained by cell area because the cells displayed similar cell areas on the nanotopographies of same height (Fig. 2C). However, as shown in Fig. 2B, the nanogratings significantly elongated the cells in comparison with the nanopillars and the flat controls, which could induce high actomyosin contractility.⁴³ The cell biomechanics measurement also implied that the nanogratings could stiffen cytoskeleton and nucleus, thus promoting cell proliferation. When nanotopography induced cell spreading and elongation worked in a synergistic manner, the cell proliferation was significantly altered. For instance, the cell proliferation on 500-1-150 was significantly higher than the flat controls ($p = 0.027$) and the cell proliferation on 500-1.9-560 was significantly lower than the flat controls ($p = 0.017$).

MWCNTs treatment of the fibroblasts at a low dosage ($0.2 \mu\text{g}/\text{cm}^2$) increased the proliferation of fibroblasts on the nanotopographies of 560 nm height as well as the flat controls, but decreased the cell proliferation on the nanotopographies of 150 nm height (Fig. 6). The observation agreed with previous *in vitro* CNT treatment studies using flat PS surfaces that low dosages of MWCNT ($0.02\text{--}0.2 \mu\text{g}/\text{cm}^2$) enhanced the proliferation of fibroblasts.^{46, 47} A recent study found that soft substrates lowered cell membrane tension, favoring polystyrene nanoparticle uptake via endocytosis compared to stiffer substrates. It was also suggested that because stiffer substrates increased the cell area, stiffer substrates resulted in a higher total cellular uptake.⁴⁹ In our study, although the shallow nanotopographies did not enlarge the cell area (Fig. 2C) yet significantly increased the nuclear volume compared to the flat controls³¹, which was postulated to increase nuclear accessibility to MWCNTs.^{50, 51} It has been revealed that CNTs could enter the cytoplasm and localize within the cell nucleus, causing cell mortality.^{45, 52} Together, the resulted increase in nuclear volume led to cell death and decreased cell viability on the 150-nm nanotopographies. By contrast, the fibroblasts were less stretched on the deeper nanopillars, accompanied with softer cytoskeleton and nucleus. The CNT-enhanced cell proliferation became evident, i.e., MWCNTs induced 15.9% increase in cell proliferation on 500-1.9-560, significantly higher than 7.6% and 7.7% increase on flat controls and 500-1-560, respectively, indicating that cell proliferation on 500-1.9-560 was more sensitive to the CNTs. The results suggested that nanotopography could modulate cell spreading and elongation, and consequently cytoskeletal and nuclear stiffness, thus altering fibroblasts proliferation in response to the CNTs.

Collagen production

We next examined the influence of nanotopography on MWCNTs induced production of collagen, especially, type I collagen, the most abundant ECM proteins robustly expressed in lung fibrosis.^{53, 54} Immunofluorescence staining qualitatively showed that collagen I production on the nanotopographies differed from the flat controls, and MWCNTs treatment increased collagen I production to various extents (Fig. 7A). Expression of collagen I was further assessed using western blot (Fig. 7B and C). In the absence of MWCNTs, collagen I production on the flat controls was similar to other nanotopographies except 500-1-150, where collagen I production was largely enhanced ($p = 0.006$). Intriguingly, MWCNTs treatment resulted in a significant increase in collagen I production on the flat controls ($p <$

0.001) and 500-1.9-560 ($p = 0.007$) but not on other nanotopographies. Moreover, compared with the untreated cells, the MWCNTs treatment induced 48.8% increase of collagen I production on 500-1.9-560, higher than 26.4% on the flat controls. The results suggested that collagen I production of the fibroblasts in response to MWCNTs were more sensitive on 500-1.9-560. We further examined the effects of MWCNTs and nanotopography on expression of the isoforms of collagen I, $\alpha 1$ and $\alpha 2$. No significant change in collagen I $\alpha 2$ expression was observed while the expression of collagen I $\alpha 1$ increased with an increase in MWCNT dose (Fig. S3 in ESI). Similarly, collagen I $\alpha 2$ expression was not sensitive to the nanotopography studied. However, the expression of collagen I $\alpha 1$ increased in the cells grown on substrates in the order, deep nanopillars 500-1.9-560, flat controls, and shallow nanogratings 500-1-150 (Fig. S4 in ESI), implying that collagen I $\alpha 1$ expression was affected by cell spreading. Compared to the nanotopography, collagen I $\alpha 1$ synthesis induced by MWCNTs was less significant. Activation of the small GTPase Rho/Rho kinase pathway stimulated cytoskeletal tension and contributed to increased collagen I synthesis in lung fibrosis.⁵⁵ Shallow nanotopographies could promote cell spreading and nanogratings could induce elongation, leading to elevated cytoskeletal stiffness that was tightly linked with cytoskeletal tension. Thereby, the basal level of collagen I was high and CNTs-induced collagen I production was not significant on the shallow nanogratings compared with that on the deep nanopillars where the cells were less spread and less stretched.

The findings had important implications. Current *in vitro* models using flat, stiff PS surfaces favored cell adhesion and spreading, thus promoting cell proliferation and collagen production, significantly deviated from the *in vivo* processes. The adhesion enhanced collagen overproduction was likely to compromise the fibrogenic response sensitivity of fibroblasts to nanomaterials, and thus the unrealistic collagen overproduction should be minimized to more accurately model *in vivo* cellular responses. The current study suggested that physiologically relevant, nanostructured substrates could increase the fibrogenic response sensitivity, thereby enabling more closely predicting the fibrogenic responses of fibroblasts to nanomaterials such as CNTs.

ROS generation

Oxidative stress has been regarded as one of the important mechanisms of nanomaterials-induced fibrogenesis.^{56, 57} ROS has been suggested to play a key role in single-walled CNT-induced transforming growth factor (TGF) activation and pulmonary fibrosis of lung fibroblasts.⁵⁸ We thereby examined the influence of nanotopography on ROS generation of the fibroblasts in response to MWCNTs. As shown in Fig. 8A, the cells expressed ROS differently on the nanotopographies compared with the flat controls, and MWCNTs enhanced ROS generation. Quantitative image analysis demonstrated that, in the absence of MWCNTs, a decrease in the height of nanotopography including both nanogratings and nanopillars elevated ROS generation, and the nanogratings facilitated ROS generation compared to the nanopillars of the same height, which was similar to the nanotopographical effects on cell proliferation. Because of the synergistic effects of shallow and anisotropic nanotopography, the ROS expression reached a peak on 500-1-150 (Fig. 8B). The MWCNTs treatment increased ROS generation on all substrates, in particular, significant increase was observed on the flat controls ($p = 0.004$) and 500-1.9-560 ($p = 0.001$). The nanotopography

effects on ROS generation of the fibroblasts in response to MWCNTs was similar to collagen I production. Among the nanotopographies, MWCNTs treatment induced most remarkable increase in ROS generation by 169% on nanopillars 500-1.9-560, higher than 108% on the flat controls.

We postulated that fibrogenic response of the fibroblasts to the CNTs was affected by both cell spreading and elongation via cell contractility, which could be modulated by altering geometry and dimensions of substrate nanotopography. Shallow nanotopography enabled the cell to confine on the substrate and thus to perceive more surface area, leading to the increase in cell spreading, nuclear volume, cytoskeletal and nuclear stiffness. Anisotropic nanotopography such as nanogratings provided continuous contact guidance to elongate the cells and further increased cellular contractility. Previous study showed that enhanced cell spreading and elongation of murine mammary epithelial cells upregulated expression of α -smooth muscle actin (α -SMA)⁵⁹, a marker of myofibroblasts that play an important role in fibrosis. Nanotopography could therefore be designed to promote cell spreading and elongation and hence cellular contractility, leading to enhanced proliferation and collagen I production.⁶⁰ For instance, nanogratings 500-1-150 enhanced cell spreading and elongation of the fibroblasts, thus resulting in the highest cell proliferation, collagen I production and ROS generation in the absence of MWCNTs. In contrast, isotropic, taller nanotopography such as nanopillars 500-1.9-560 resulted in reduced cell spreading, smaller nuclear volume, and less stiff cytoskeleton and nucleus, accompanied with lower cell proliferation and collagen I production of the fibroblasts. In the scenario where the cells and the nuclei were largely stretched, MWCNTs treatment induced a limited increase in the fibrogenic response of the fibroblasts, even causing cell death. On the other hand, when the fibroblasts were not pre-stressed, the cells became more sensitive to the CNTs and showed significant increases in cell proliferation, collagen I production and ROS generation. Taken together, by altering the geometry and dimensions, in particular the height of nanotopography, unrealistic adhesion-induced cell proliferation and collagen overproduction of fibroblasts could be alleviated and thus the fibrogenic response sensitivity of the fibroblasts to the CNTs was enhanced. Because nanotopography was of physiological relevance, there was a good reason to believe that toxicological study using nanostructured substrate could more closely mimic *in vivo* phenotypes and responsiveness.

Conclusion

The present study investigated the influence of geometry and dimensions (height) of nanotopography on cell proliferation, collagen I production and ROS generation of normal human lung fibroblasts in response to MWCNTs and suggested that the fibrogenic response was affected by the underneath substrate nanotopography. Analyses of cell spreading, and cytoskeletal and nuclear stiffness of the fibroblasts suggested that nanotopography could effectively modulate cellular response to the MWCNTs. By altering the geometry and height of nanotopography, the cells could be largely stretched, accompanied with enhanced cell proliferation and collagen I production, and consequently, undermining fibrogenic response sensitivity of the fibroblasts to the CNTs. By contrast, the cell spreading and cytoskeletal tension could be reduced using tall, isotropic nanotopography, which enhanced the cellular sensitivity to the CNTs. This study highlighted the important role of cell-nanotopography

interactions in fibrogenic response of the fibroblasts to nanomaterials, and contributed to development of physiologically relevant *in vitro* models for nanotoxicology study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. SEM micrographs of the PDMS substrates. Nanotopography was labelled by using a combination of the feature size, spacing and height. For instance, “500-1.9-560” refers to the nanopillars of 500 nm in diameter, 1.9 times diameter in center-to-center spacing and 560 nm in height. The inset provides the enlarged image. Scale bars are 1 μm .

Fig. 2.

Cell spreading on the PDMS substrates. (A) Fluorescent images of the fibroblasts on the PDMS substrates. The cells were stained for F-actin in green and nuclei in blue. The white arrows indicate the direction of nanogratings. Scale bars are 1 μm . (B) Cell roundness and (C) cell area of the fibroblasts grown on the PDMS substrates after 24 h of culture. *: significant difference ($p < 0.05$) from the flat controls; +: significant difference ($p < 0.05$) between groups; NS: not significant.

Fig. 3. SEM micrographs of fibroblasts on the PDMS substrates. Black arrows and red arrowheads point to the lamellipodia and filopodia of fibroblasts, respectively. Scale bars are 1 μm .

Fig. 4.
 (A) Cytoskeletal and (B) nuclear stiffness of the fibroblasts on the PDMS substrates. *: significant difference ($p < 0.05$) from the flat controls; +: significant difference ($p < 0.05$) between groups.

Fig. 5. Collagen expression of fibroblasts treated with MWCNTs. (A) Immunoblotting of collagen I. Fibroblasts were treated with MWCNTs at the doses of 0.06, 0.2 and 0.6 $\mu\text{g}/\text{cm}^2$ for 48 h. β -actin was used as the loading control. (B) Densitometry analysis of collagen I expression based on the results of (A).

Fig. 6. Proliferation of fibroblasts on the PDMS substrates. The cells were either treated with MWCNTs for 48 hr (labelled as MWCNTs in the plot) and without MWCNTs treatment as the controls (CTRL). *: significant difference ($p < 0.05$) from the flat controls; +: significant difference ($p < 0.05$) between groups.

Fig. 7.

Nanotopography affected collagen I expression of fibroblasts in response to MWCNTs treatment at $0.2 \mu\text{g}/\text{cm}^2$. (A) Immunofluorescent staining of the cells exposed to MWCNTs. Collagen I was stained in green and the nucleus was stained with DAPI in blue. Scale bars are $50 \mu\text{m}$. (B) Immunoblotting of collagen I expression. β -actin was used as the loading control. The bands labelled with (-) and (+) were from the cells without and with MWCNTs treatment, respectively. (C) Densitometry analysis of collagen I expression. The cells without and with MWCNTs treatment were labelled as CTRL (the controls) and MWCNTs, respectively. *: significant difference ($p < 0.05$) from the flat controls; +: significant difference ($p < 0.05$) between groups.

Fig. 8. Nanotopography modulated ROS generation of fibroblasts in response to MWCNTs. (A) Fluorescent images of fibroblasts on nanotopographies. Fibroblasts were either treated with MWCNT for 16 h (MWCNTs) or without MWCNTs treatment (CTRL). ROS was stained with DCF-DA in green and nuclei were stained with DAPI in blue. Scale bars are 100 μm. (B) Quantitative ROS images analysis from at least ten separate fields. ROS production was normalized to the mean value of flat group without MWCNTs treatment. +: significant difference ($p < 0.05$) between groups.