

Multiwall Carbon Nanotubes Mediate Macrophage Activation and Promote Pulmonary Fibrosis Through TGF- β /Smad Signaling Pathway

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Multiwall carbon nanotubes (MWCNTs) have been widely used in many disciplines due to their unique physical and chemical properties, but have also raised great concerns about their possible negative health impacts, especially through occupational exposure. Although recent studies have demonstrated that MWCNTs induce granuloma formation and/or fibrotic responses in the lungs of rats or mice, their cellular and molecular mechanisms remain largely unaddressed. Here, it is reported that the TGF- β /Smad signaling pathway can be activated by MWCNTs and play a critical role in MWCNT-induced pulmonary fibrosis. Firstly, *in vivo* data show that spontaneously hypertensive (SH) rats administered long MWCNTs (20–50 μ m) but not short MWCNTs (0.5–2 μ m) exhibit increased fibroblast proliferation, collagen deposition and granuloma formation in lung tissue. Secondly, the *in vivo* experiments also indicate that only long MWCNTs can significantly activate macrophages and increase the production of transforming growth factor (TGF)- β 1, which induces the phosphorylation of Smad2 and then the expression of collagen I/III and extracellular matrix (ECM) protease inhibitors in lung tissues. Finally, the present *in vitro* studies further demonstrate that the TGF- β /Smad signaling pathway is indeed necessary for the expression of collagen III in fibroblast cells. Together, these data demonstrate that MWCNTs stimulate pulmonary fibrotic responses such as fibroblast proliferation and collagen deposition in a TGF- β /Smad-dependent manner. These observations also suggest that tube length acts as an important factor in MWCNT-induced macrophage activation and subsequent TGF- β 1 secretion. These *in vivo* and *in vitro* studies further highlight the potential adverse health effects that may occur following MWCNT exposure and provide a better understanding of the cellular and molecular mechanisms by which MWCNTs induce pulmonary fibrotic reactions.

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1. Introduction

Carbon nanotubes (CNTs) are recently developed, promising nanomaterials due to their remarkable mechanical, electrical, and magnetic properties. They are anticipated to revolutionize the fields of electronics, structural engineering, and medicine.^[1-3] With the increasing production of single-wall CNTs (SWCNTs) and multiwall CNTs (MWCNTs), there is an accompanying increase in the potential for human exposure, especially in occupational settings.^[4-6] It is rather remarkable that some of the physical properties of MWCNTs that make them desirable for electronics, engineering, and medicine may also augment their toxic potential relative to larger particles or even other types of nanoparticles. Some of these properties include altered surface chemistry compared with larger particles of the same material, a high surface area to volume ratio, a high length-to-width aspect ratio, and a high degree of biopersistence. Indeed, the biosafety issues of CNTs have attracted enormous attention in recent years. Wang et al. have reported that chronic exposure to SWCNTs causes malignant transformation of human lung epithelial cells.^[7] Meng et al. have studied the effect of different MWCNTs on the neuron growth factor (NGF)-induced differentiation of PC12 cells, which is correlated to the metal impurities or tube length of CNTs.^[8,9] Some reviews have also raised many concerns about the nanosafety of CNTs and the possible mechanism of carbon nanotube-induced toxicity.^[10,11] Therefore, it will be necessary to systematically assess the potential adverse effects of CNTs on human health and the environment during their applications.

A number of studies have addressed the potential of CNTs to cause pulmonary fibrosis, the scarring of lung tissue caused by an increase in fibroblasts and their collagen deposits.^[12] SWCNTs or MWCNTs administered by intratracheal instillation or pharyngeal aspiration cause inflammation in the lungs of rats and mice, as well as formation of granulomas and/or fibrotic responses.^[13-17] Recent studies showed that fibrotic reactions in the lungs of mice exposed to MWCNTs were associated with increased levels of transforming growth factor (TGF)- β 1 in bronchoalveolar lavage (BAL) fluid.^[18,19] However, the detailed cellular and molecular mechanisms by which MWCNTs cause inflammation and fibrosis in the lung remain largely unaddressed.

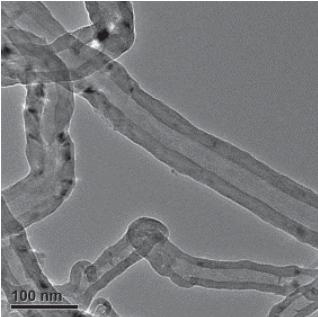
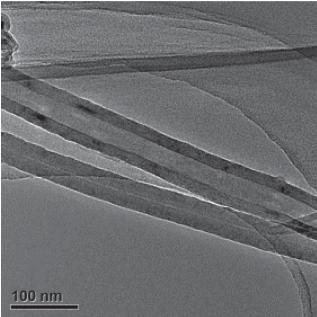
Because CNTs differ in their physical and chemical properties such as diameter, length and impurities, there are disparities in the experimental results for nanotoxicity that could render the mechanism of CNT-induced fibrogenesis elusive. Several studies have demonstrated that different dispersions of MWCNTs were associated with their pulmonary toxicity.^[19-21] Our previous studies suggest that metal impurities of CNTs or interactions between CNTs and protein play important roles in CNT-caused cytotoxicity.^[22-24] Importantly, a recent discovery showed that the diameter and rigidity of MWCNTs are critical factors in mesothelial injury and carcinogenesis.^[25] Palomaki et al. also reported that long, needle-like but not short or long-tangled MWCNTs can activate the NLRP3 inflammasome

in macrophages through a mechanism similar to that of asbestos.^[26] Indeed, long MWCNTs induce frustrated phagocytosis and granuloma formation in the mesothelium.^[27] The “length-dependent theory” is useful to explain CNT-caused inflammation and mesothelioma in the parietal pleura.^[28] However, to date, few studies have examined whether the tube length of CNTs contributes to CNT-caused pulmonary fibrosis. Therefore, to determine whether fiber length can play a critical role in MWCNT-induced fibrogenesis, more *in vivo* and *in vitro* experiments are still urgently needed.

Due to increasing longevity and the prevalence of contributing factors such as obesity, hypertension is already a highly prevalent cardiovascular risk factor worldwide. We previously reported that an acute exposure of the lungs of spontaneously hypertensive (SH) rats to SWCNTs by a non-surgical intratracheal instillation induced acute pulmonary and cardiovascular responses, which were associated with an increased level of tumor necrosis factor- α (TNF- α) in the BAL fluid of SH rats.^[29] However, the long-term effects are not clear. Given that pulmonary fibrosis is a chronic disease associated with multiple cellular origins involving complex and interrelated signaling pathways, it is important to determine whether pulmonary fibrosis has the potential to crosstalk with other diseases, especially some chronic diseases. Hypertension is associated with multiple functional and structural cardiovascular alterations, which include myocardial fibrosis induced by local production of TGF- β 1. Therefore, it seems that pulmonary fibrosis would be easier to induce in an experimental model of hypertension. However, to date, there has been no report analyzing the risk of pulmonary fibrosis generated by CNTs in SH rats.

Therefore, in the current work, we focus on molecular and cellular mechanisms by which MWCNT may induce lung fibrosis after long-term exposure. We chose SH rats as a susceptible model in our *in vivo* studies and used two types of MWCNTs (see Table 1), short MWCNTs (0.5–2 μ m) and long MWCNTs (20–50 μ m), to study the role of tube length in MWCNT-induced pulmonary fibrosis. Importantly, our *in vivo* studies showed that long MWCNTs, but not short MWCNTs, caused fibrotic responses including granuloma formation, collagen deposition and fibroblast proliferation. Moreover, we analyzed the role of MWCNTs in activating the TGF- β /Smad signaling pathway by measuring the production of TGF- β 1 from MWCNT-stimulated macrophages and by detecting the mRNA expression of TGF- β 1, T β RII and Smad2/3/7 using real-time polymerase chain reaction (PCR) and phosphorylation of Smad2 by immunoblotting and immunohistochemistry. Finally, we checked whether MWCNTs could promote the expression of collagen III *in vitro* through activating the TGF- β /Smad signaling pathway. Such a detailed molecular characterization of MWCNT-induced TGF- β /Smad signaling activation, to our knowledge, has not previously been achieved, and this study provides an important insight into the cellular and molecular mechanisms involved in MWCNT-induced granuloma formation and fibrotic responses.

Table 1. Specifications of MWCNTs.

	Short MWCNTs	Long MWCNTs
Outside Diameter (OD, nm)	≈50	≈50
Length (μm)	0.5–2	20–50
CNT Purity (wt%)	>95	>95
Specific surface areas (SSA, m ² g ⁻¹)	>40	>50
TEM Morphology (Measure bar 100 nm)		

Note: Representative transmission electron microscopy (TEM) images of short and long MWCNTs were taken with a Tecnai G220 S-TWIN transmission electron microscope.

2. Results and Discussion

2.1. Long but Not Short MWCNTs Significantly Increase Fibrosis in the Lungs of MWCNT-Exposed SH Rats

It has been reported that long MWCNTs induce frustrated phagocytosis and granuloma formation in the mesothelium.^[27] Here, we asked whether fiber length could also play a critical role in MWCNT-induced fibrogenesis in the lung. We answered this question by administering short or long MWCNTs to the lungs of SH rats by a non-surgical intratracheal instillation and comparing their fibrotic effects 1 day, 7 days or 30 days after exposure.

Granulomas were strongly induced in the lung tissues of SH rats exposed to long MWCNTs 30 days after exposure. In contrast, we could not find any granuloma in the group treated with short MWCNTs (Figure 1A). Similarly, 30 days post-exposure to the long MWCNTs, the deposition of collagen was easily observed in both granulomatous regions and the areas distant from granulomas, as established by immunohistochemistry examination (Figure 1B) and Sirius Red staining (Figure 1C, D). However, there is no such phenomenon 30 days after exposure to short MWCNTs (Figure 1B–D). It is well known that fibroblasts are responsible for the production of collagen. Thus, we analyzed the expression of fibroblast-specific protein-1 (FSP-1), a fibroblast marker, by immunohistochemistry, and found that long MWCNTs clearly increased the number of FSP-1 positive cells even at only one day post-exposure. However, short MWCNTs caused only a weak elevation at 30 days post-exposure (Figure 1E, F). Moreover, it appears that the granuloma formation, collagen deposition and fibroblast proliferation in

the lung tissues caused by long MWCNT exposure progressed with time.

Taken together, our data show that long MWCNTs but not short MWCNTs cause obvious granuloma formation and fibrotic effects, which include an increase in fibroblasts and their collagen deposits in lung tissues of SH rats. These results suggest that the fibrotic effect of MWCNTs is length-dependent. It has been reported that the effect of MWCNTs on mesothelial injury was length- and diameter-dependent.^[25,27] Yamashita et al. also showed that long and thick MWCNTs, but not short and thin MWCNTs, can cause DNA damage and severe inflammatory effects.^[30] However, there are still some uncertainties about whether the length of MWCNTs could play an important role in MWCNT-caused lung toxicity. Muller et al. reported that intratracheal instillation of 0.5–5 mg of long (6 μm) and short (0.7 μm) non-functionalized MWCNTs led to long persistence of inflammation and fibrosis of lung tissue, without significant length-dependent differences.^[17] Perhaps these various results are due to the different lengths and diameters of the MWCNTs they chose.^[31] Given that MWCNTs with a diameter of 50 nm caused the most significant mesothelial injury,^[25] in our study we chose short (0.5–2 μm) and long (20–50 μm) MWCNTs whose diameters were all about 50 nm. Our findings suggest that short MWCNTs cause less lung injury than long MWCNTs. Herein, “fiber-like” pathogenic behavior is expected based on the available data. In this case, our data suggest that long and rigid MWCNTs should be avoided for in vivo applications not only for their mesothelial potential but also for their promotion of pulmonary fibrosis.

As is known, pulmonary fibrosis is characterized by excessive accumulation of extracellular matrix (ECM) in the lung tissue. Accumulation of ECM in fibrotic diseases usually results from elevated mRNA levels of fibrillar collagens due to increased transcriptional activation. Moreover, inhibitors of ECM degradation may also play important roles. ECM is mainly degraded via two distinct pathways: the matrix metalloproteinases (MMP) degrading pathway and the plasminogen activators (PA)/plasmin proteolytic axis.^[32,33] Both pathways have specific inhibitors: tissue inhibitors of metalloproteinases (TIMP) and plasminogen activator inhibitors (PAI), respectively.^[34] A previous report has described PAI-1 induction in the lungs of mice exposed to CNTs as a potential cardiovascular risk factor.^[35] However, how TIMP-1 or PAI-1

2.2. Long MWCNTs Cause Collagen Deposition by Upregulating Both ECM Protease Inhibitors and Collagen Type I/III mRNA Transcription in the Lungs of MWCNT-Exposed SH Rats

As is known, pulmonary fibrosis is characterized by excessive accumulation of extracellular matrix (ECM) in the lung tissue. Accumulation of ECM in fibrotic diseases usually results from elevated mRNA levels of fibrillar collagens due to increased transcriptional activation. Moreover, inhibitors of ECM degradation may also play important roles. ECM is mainly degraded via two distinct pathways: the matrix metalloproteinases (MMP) degrading pathway and the plasminogen activators (PA)/plasmin proteolytic axis.^[32,33] Both pathways have specific inhibitors: tissue inhibitors of metalloproteinases (TIMP) and plasminogen activator inhibitors (PAI), respectively.^[34] A previous report has described PAI-1 induction in the lungs of mice exposed to CNTs as a potential cardiovascular risk factor.^[35] However, how TIMP-1 or PAI-1

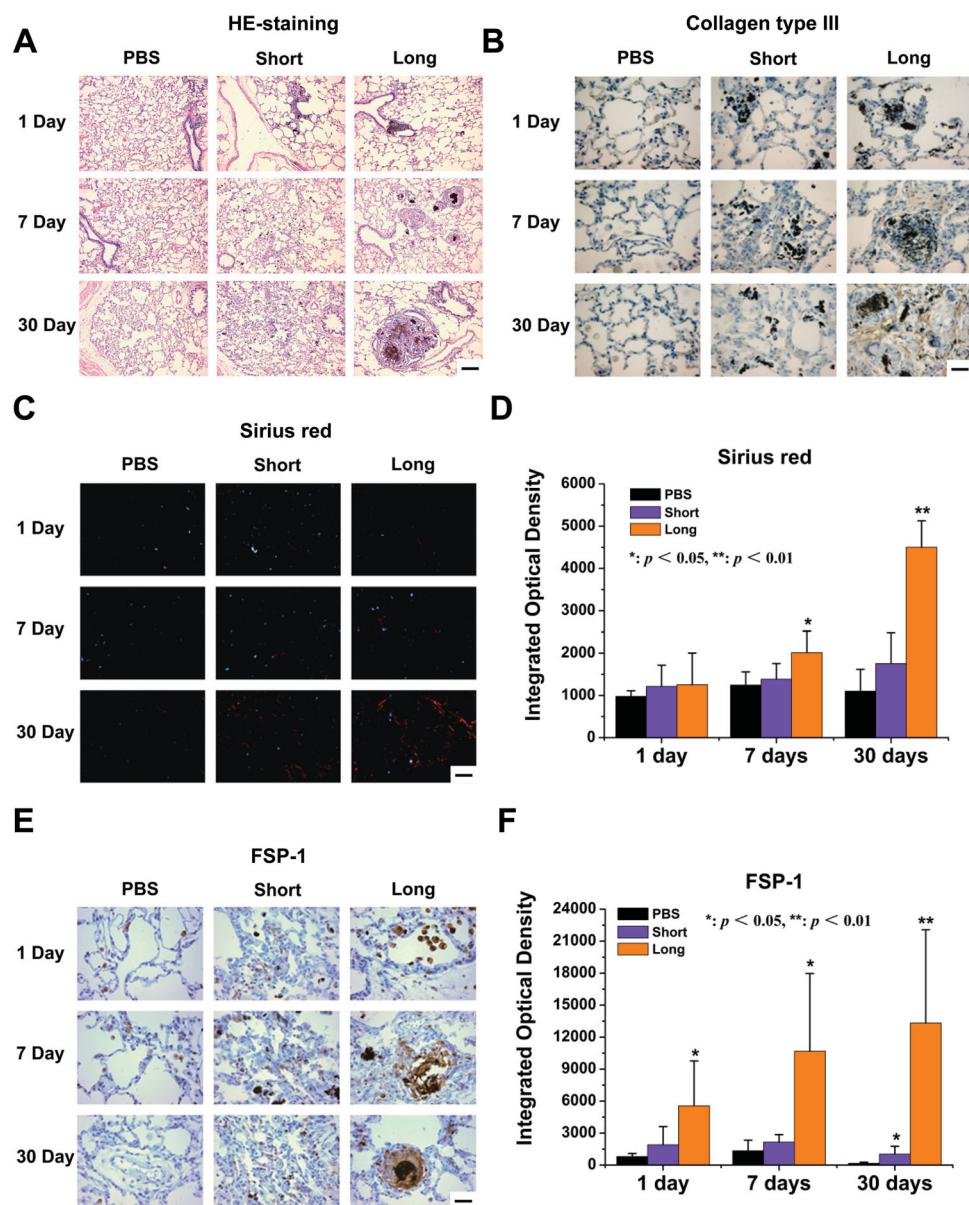


Figure 1. Comparison of the fibrotic effect of different MWCNTs in the lungs of spontaneously hypertensive rats. SH rats were exposed by intratracheal instillation to short or long MWCNTs (0.6 mg/rat) and lung tissues were obtained 1, 7, or 30 days after exposure. Granuloma was detected by A) HE-staining, and B) production of collagen type III in lung tissue was determined by immunohistochemistry at the indicated time post-exposure. C) The content of collagen type I/III in lung tissues was determined by Sirius Red staining and D) the optical density was integrated by Leica's Microscope Software. E) FSP-1 (a marker of fibroblasts) positive cells in lung tissue were observed by immunohistochemistry and F) the optical density was integrated by Leica's Microscope Software. All values are means \pm SD from three independent analyses. Scale bar as follows: 100 μ m (A and C), 50 μ m (B and E).

is regulated in the lungs of SH rats after long-term exposure to MWCNTs is still unknown.

Thus, to elucidate the possible mechanisms of MWCNT-caused collagen deposition, we analyzed the mRNA expression levels of ECM protease inhibitors and collagen I/III in the lung tissue of SH rats exposed by intratracheal instillation to short or long MWCNTs for 1, 7, or 30 days. Real-time polymerase chain reaction (PCR) results showed that the mRNA expression of both TIMP-1 and PAI-1 was significantly increased by long MWCNT exposure for 7 or 30 days, but not 1 day (Figure 2A). However, it seems that there is

selectivity for short MWCNTs in the regulation of TIMP-1 and PAI-1. Short MWCNTs clearly promoted TIMP-1 transcription but had a limited effect on PAI-1 mRNA expression (Figure 2A). Except for detecting these two ECM protease inhibitor genes, the mRNA transcriptions of collagen I/III were also checked using real-time PCR. In accordance with our data from immunohistochemistry examination (Figure 1B) and Sirius Red staining (Figure 1C, D), collagen I/III mRNA transcription was time-dependently upregulated by long but not short MWCNT treatment (Figure 2B). Taken together, our data suggest that MWCNTs cause collagen deposition

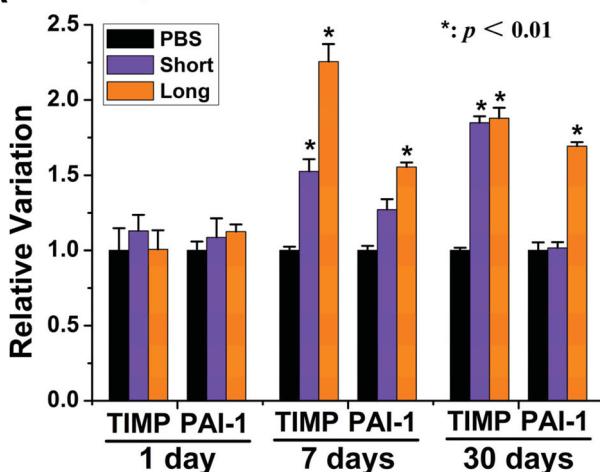
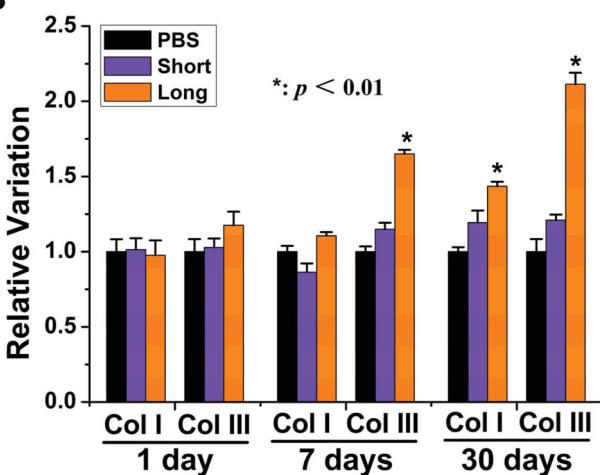
A ECM protease inhibitors mRNA level**B Col I/III mRNA level**

Figure 2. Effects of MWCNT exposure on expression of ECM protease inhibitors and collagen type I/III in vivo. SH rats were exposed by intratracheal instillation to short or long MWCNTs (0.6 mg/rat) and lung tissues were prepared for RNA isolation at 1, 7, or 30 days post-exposure. Real time quantitative PCR was performed using 2 μ g of total RNA for A) two ECM protease inhibitor genes, TIMP and PAI-1 or B) collagen type I/III. All values are means \pm SD from three independent analyses.

by upregulating both ECM protease inhibitors and collagen type I/III mRNA transcription in the lungs of MWCNT-exposed SH rats. Moreover, the fiber length of MWCNTs might play a critical role in MWCNT-induced transcriptional activation.

Several studies have reported that MWCNTs upregulate the expression of collagens.^[18,36,37] However, they only detected the protein level of collagen; whether collagen mRNA transcription could be affected by MWCNT treatment is unknown. Moreover, the effect of MWCNTs on the expression of the ECM protease inhibitor gene has not been systematically studied. Importantly, our study indicated that long MWCNTs contributed to fibrosis not only by enhancing collagen transcription but also by inhibiting ECM degradation, by inducing transcription of protease inhibitors

such as TIMP-1 and PAI-1. As is known, the TGF- β /Smad signaling pathway is responsible for the transcriptional activation of collagen I/III, TIMP-1 and PAI-1. Thus exploration of whether MWCNTs cause fibrosis through activating the TGF- β /Smad signaling pathway in lung tissues is urgently needed.

2.3. Long MWCNTs Stimulate the Production of TGF- β 1 by Mediating Activation of Macrophages and Causing More Damage to the Bronchiolar Epithelium

Fibrosis is a disease of multiple cellular origins involving complex and interrelated signaling pathways. The production of inflammatory cytokines and chemokines from activated macrophages is a hallmark of the pulmonary response to fibrogenic fibers.^[12] Thus, in order to figure out the interaction between MWCNTs and macrophages and the effect of MWCNTs on macrophage activation, we first examined the cellular uptake of different MWCNTs by alveolar macrophages in BAL fluid or lung tissues. From **Figure 3A**, both short and long MWCNTs can be observed in alveolar macrophages from BAL fluid. Interestingly, images from alveolar macrophages of SH rats exposed to long MWCNTs show that incomplete or frustrated phagocytosis occurred. We also detected the distribution of MWCNTs in the lung tissue, and the results showed that both MWCNTs could be taken up mainly by alveolar macrophages localized in the pulmonary alveoli (Figure 3B). Together, our data suggested that alveolar macrophages were responsible for the clearance of administered MWCNTs from the lung.

Among the mediators involved in tissue fibrosis, TGF- β is considered a key molecule in the activation of the fibrotic program. Meanwhile, TNF- α is considered a central mediator of chronic inflammatory diseases. It has been reported that both TGF- β 1 and TNF- α can be stimulated by inflammatory stimuli in macrophages.^[38,39] Therefore, we next detected the production of TGF- β 1 and TNF- α from MWCNT-stimulated macrophages. As shown in **Figure 4A**, the production of TGF- β 1 was significantly stimulated by long MWCNT treatment for 1 day. However, the short treatment group had no effect on the level of TGF- β 1 in BAL fluid. By contrast, TNF- α levels increased at 7 days after but not 1 day or 30 days after exposure to short or long MWCNTs (Figure S1, Supporting Information). Interestingly, we also used real-time PCR to detect the mRNA expression of TGF- β 1 in lung tissues, and found out that TGF- β 1 transcription was upregulated by MWCNTs only after 7 days of exposure, but not after 1 day or 30 days, and that long MWCNTs enhanced TGF- β 1 transcription more significantly than short MWCNTs (Figure 4B).

In addition, we looked at whether TGF- β 1 could be secreted by MWCNT-stimulated macrophages by immunohistochemistry. As shown in Figure 4C, TGF- β 1 could be clearly observed in MWCNT-stimulated alveolar macrophages. Moreover, it seems that TGF- β 1 produced by MWCNT-stimulated alveolar macrophages could be observed only after 7 days of exposure, but not after 1 day or 30 days, and that more TGF- β 1 could be generated by alveolar macrophages exposed to long MWCNTs (Figure 4D).

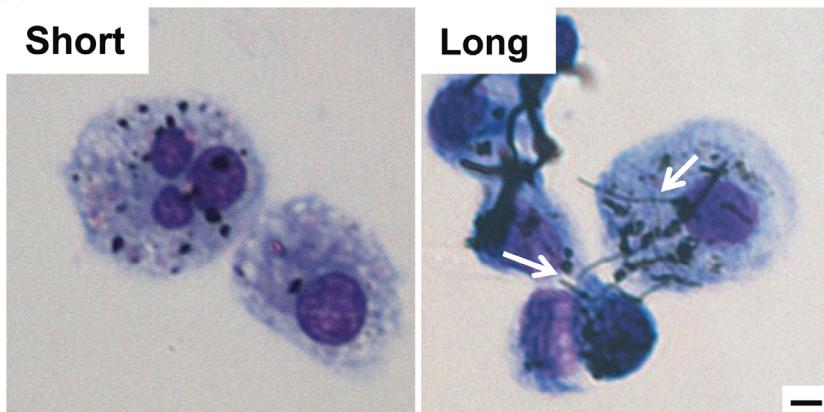
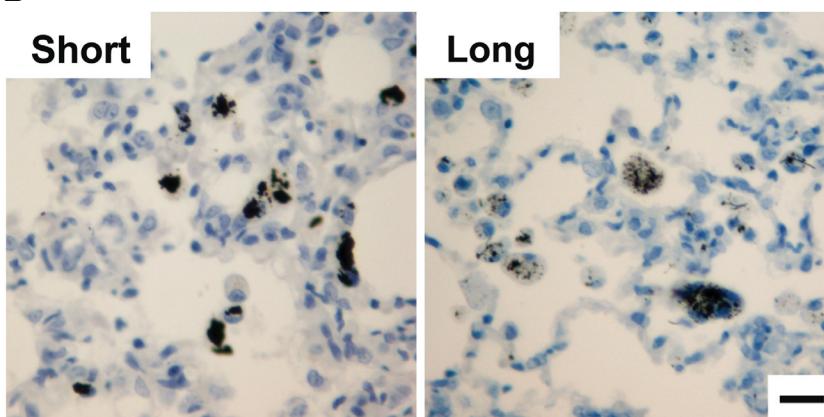
A**B**

Figure 3. Representative optical microscope images to show the cellular uptake of MWCNTs by alveolar macrophages *in vivo*. A) SH rats were exposed by intratracheal instillation to short or long MWCNTs (0.6 mg/rat) and BAL fluid was obtained 7 days after exposure. Uptake of MWCNTs by alveolar macrophages in BAL fluid was observed by optical microscopy (100 \times). B) Representative regions in lung tissues of short or long MWCNT-exposed SH rats. Both short and long MWCNTs could be phagocytosed by alveolar macrophages 7 days after exposure. The white arrows point to CNTs extending outside the macrophage in the process of incomplete or frustrated phagocytosis. Scale bar as follows: 5 μ m (A), 30 μ m (B).

Together, these results suggest that MWCNT-stimulated alveolar macrophages are responsible for TGF- β 1 production in the lung tissue of SH rats exposed to MWCNTs for 7 days.

In fact, several studies have already reported that TGF- β 1 could be stimulated in the BAL fluid of MWCNT-administered mice.^[18–23] However, the mechanisms of MWCNT-caused TGF- β 1 secretion into BAL fluid are still unknown. Therefore we used real-time PCR and immunohistochemistry to detect TGF- β 1 production and found that MWCNTs increased TGF- β 1 production only after 7 days of exposure (Figure 4B–D); however, upregulation of TGF- β 1 secretion into BAL fluid could only be detected after 1 day of exposure to long MWCNTs (Figure 4A).

It has been previously reported that protein expression of TGF- β 1 is confined to the bronchiolar epithelium.^[40] Consistent with this study, we also observed constitutively expressed TGF- β 1 near the bronchiolar epithelium (Figure 5A). This constitutive TGF- β 1 expression near the bronchiolar epithelium might be significant for the constitutive expression of collagen III (Figure 5C), which

is mediated by phosphorylated Smad2 (Figure 5B), and it might also be necessary for the physiological function of the bronchiolar epithelium. Moreover, as shown in Figure 5, we observed that some cell flakes appeared in the bronchus of SH rats exposed to long MWCNTs but not short MWCNTs for 1 day. We speculate that long MWCNTs might cause more damage to the bronchiolar epithelium than short MWCNTs, and this damage might induce the release of TGF- β 1 from the bronchiolar epithelium, which may recruit fibroblast cells and cause some pulmonary inflammations. Therefore, MWCNT-caused release of TGF- β 1 from the bronchiolar epithelium may be one of the mechanisms of MWCNT-caused TGF- β 1 secretion into BAL fluid. Taken together, these results suggest that TGF- β 1 could be induced in alveolar macrophages or released from the bronchiolar epithelium by MWCNT exposure and play an important role in pulmonary fibrosis.

2.4. Activation of the TGF- β /Smad Signaling Pathway in the Lung Tissues of MWCNT-Exposed SH Rats

TGF- β , a primary mediator of collagen deposition during fibrogenesis, signals through two types of membrane-bound serine/threonine kinase receptors and intracellular Smad proteins.^[41,42] Ligand binding results in the formation of the receptor heteromeric complex consisting of TGF- β type I and TGF- β type II receptors, phosphorylation of R-Smad

(receptor-regulated Smads: Smad2/3), accumulation of the Smad2/3-Smad4 complex in the nucleus, and (ultimately) modulation of gene expression. Our above studies have shown that MWCNTs could stimulate TGF- β 1 secretion in the lung by activation of alveolar macrophages, and that the fiber length of MWCNTs plays a critical role in MWCNT-stimulated production of TGF- β 1. However, to date, the effect of MWCNTs on Smad2 phosphorylation has not been directly demonstrated. Thus, to determine whether MWCNTs could activate the TGF- β /Smad signaling pathway in the lung tissue of SH rats, we examined Smad phosphorylation in fibroblast cells.

As shown in Figure 6A, TGF- β 1 significantly induced Smad2 phosphorylation in a time-dependent manner in fibroblast cells *in vitro*. We showed above that MWCNTs could stimulate TGF- β 1 secretion in the lung by activation of alveolar macrophages. To determine whether the TGF- β 1 secreted by alveolar macrophages could stimulate Smad2 phosphorylation in the lung, we used immunoblotting and immunohistochemistry to detect Smad2 phosphorylation

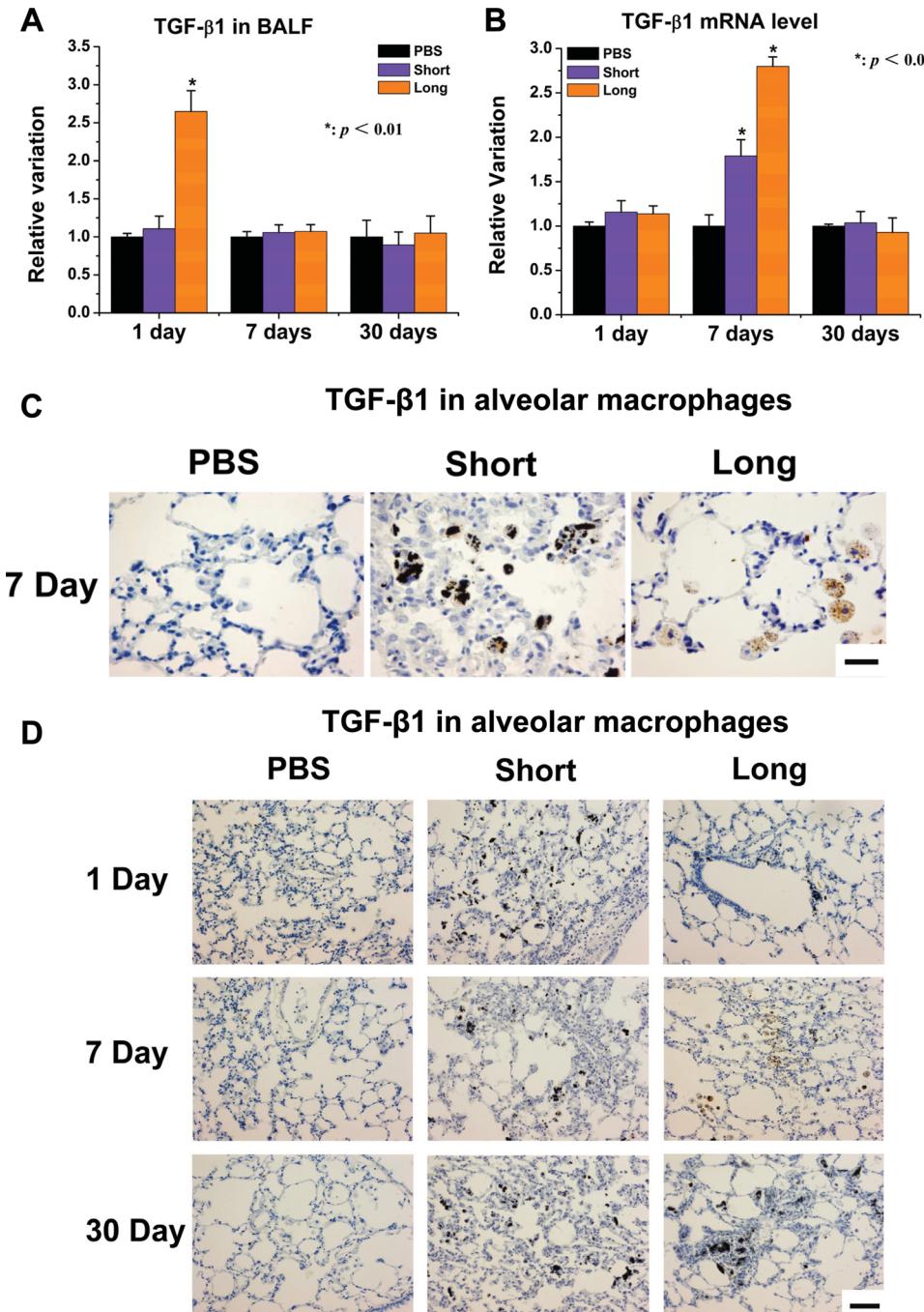


Figure 4. Effects of MWCNTs on the production of TGF- β 1 in lung tissues of SH rats. SH rats were exposed by intratracheal instillation to short or long MWCNTs (0.6 mg/rat) and BAL fluids or lung tissues were obtained at 1, 7 and 30 days post-exposure, respectively. A) ELISA analysis was used to analyze the contents of TGF- β 1 in BAL fluid. B) Real-time PCR was performed to determine the mRNA expression level of TGF- β 1 in lung tissue. C) Immunohistochemistry was employed to detect TGF- β 1 in lung tissues of SH rats exposed to short or long MWCNTs for 7 days. Scale bar 50 μ m. D) Immunohistochemistry was employed to detect TGF- β 1 in lung tissues of SH rats exposed to short or long MWCNTs for the indicated time. Scale bar 100 μ m. All values were normalized according to the PBS control. All values are means \pm SD from three independent analyses.

in lung tissues of SH rats exposed to MWCNTs for 7 days. Both immunoblotting and immunohistochemistry results showed that Smad2 phosphorylation was significantly enhanced by MWCNTs in lung tissues of SH rats 7 days after intratracheal instillation exposure (Figure 6B,C). As shown in Figure 6C, long MWCNTs caused a much higher Smad2

phosphorylation level than short MWCNTs. Moreover, our in vitro experiments also indicated that long but not short MWCNT pre-treatment significantly promoted TGF- β -induced Smad2 phosphorylation in NIH 3T3 cells (Figure 7).

Furthermore, we examined the transcription of several critical genes in this pathway, such as T β RII, R-Smads

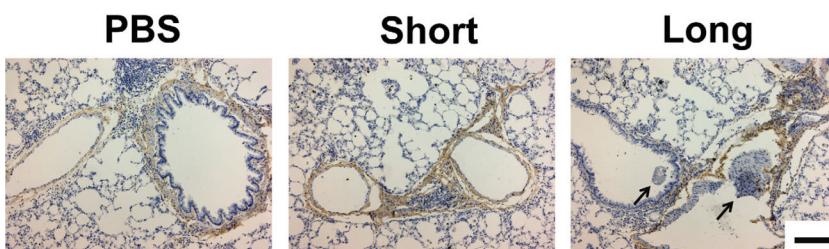
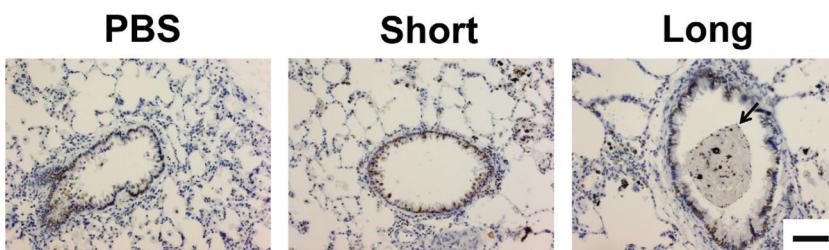
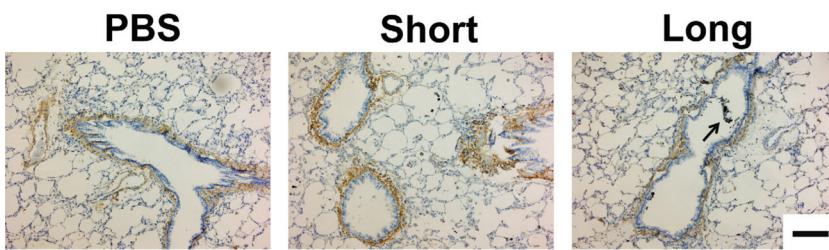
A TGF- β 1 near the bronchus**B Smad2 phosphorylation****C Collagen type III**

Figure 5. High level of TGF- β 1 expression, Smad2 phosphorylation and Collagen type III production near the bronchus in lung tissues of SH rats. SH rats were exposed by intratracheal instillation to short or long MWCNTs (0.6 mg/rat) and lung tissues were obtained 1 day after exposure. Immunohistochemistry was employed to detect A) TGF- β 1 expression, B) Smad2 phosphorylation and C) Collagen type III production near the bronchus in lung tissues of SH rats. The black arrows point to cell flakes appeared in the bronchus. Scale bar as follows: 100 μ m (A and C), 50 μ m (B).

(Smad2/3) and I-Smad (inhibitory Smad, Smad7). The results showed that both short and long MWCNTs increased the mRNA expression of T β RII (Figure 6D) and Smad3 (Figure 6E), which all contribute to activation of the TGF- β /Smad signaling pathway. However, for Smad2 and Smad7 mRNA expression, these two kinds of MWCNTs have different effects. As shown in Figure 6E, short MWCNTs have no effect on the Smad2 mRNA level, but increase expression of Smad7, while long MWCNTs inhibit both Smad2 and Smad7 mRNA transcription. Smad7 has been well established to be a key negative regulator of TGF- β signaling.^[43,44] Therefore, downregulation of Smad7 caused by long MWCNTs also contributes to the stimulation of the TGF- β /Smad signaling pathway.

Taken together, our data suggest that MWCNTs could cause TGF- β /Smad2 signaling activation in fibroblasts not only by stimulating TGF- β 1 secretion from macrophages (indirectly), but also by interacting with fibroblasts (directly). A recent study showed that MWCNT attenuates bone morphogenetic protein (BMP)/Smad1 signaling by

binding to BMP receptor 2.^[45] It would be interesting for us to study whether various MWCNTs could regulate TGF- β signaling through their interactions with TGF- β receptors. However, in this study, we focus on the interaction between MWCNTs and macrophages, which promoted secretion of TGF- β from macrophages serving as a critical paracrine stimulus to fibroblasts. To further explore the necessary role of paracrine TGF- β in MWCNTs-induced collagen III expression, we next employed in vitro co-culture experiments.

2.5. Long MWCNTs Induce Production of Collagen III in a TGF- β /Smad Dependent Manner In Vitro

It is believed that TGF- β secreted from macrophages serves as a critical paracrine stimulus to fibroblasts to promote the transformation of fibroblasts into myofibroblasts. Myofibroblasts secrete collagens to deposit in the matrix, resulting in fibrosis. Indeed, we detected the effect of TGF- β 1 on NIH 3T3 cell proliferation. As shown in Figure S2, Supporting Information, TGF- β 1 increases the proliferation of NIH 3T3 cells dose-dependently. Therefore, in order to further illustrate the critical role that TGF- β secreted from MWCNT-stimulated macrophages could play in the promotion of fibroblast proliferation and collagen deposition, we similarly investigated whether TGF- β 1 secreted from MWCNT-stimulated RAW264.7 cells could affect the function

of NIH 3T3 fibroblast cells by co-culturing NIH 3T3 cells with RAW264.7 cells in vitro.

We first examined the effect of MWCNTs on RAW264.7 and NIH 3T3 cell viability. We found that long MWCNTs caused no cytotoxicity to either RAW264.7 or NIH 3T3. Moreover, it seems that long MWCNTs can promote proliferation of RAW264.7. However, short MWCNTs downregulate cell viability of both RAW264.7 and NIH 3T3 at high concentration (Figure 8A, B).

Next, we analyzed MWCNT-stimulated production of TGF- β 1 and TNF- α in RAW264.7 cells by monitoring the mRNA expression of TGF- β 1 and TNF- α after treatment by short or long MWCNTs for 24 h. Real-time PCR results showed that the mRNA levels of both TGF- β 1 and TNF- α were more obviously upregulated by long MWCNT treatments than by short MWCNTs (Figure 8C,D). We also used the ELISA assay to determine the effect of MWCNTs on the levels of secreted TGF- β 1 and TNF- α in the culture medium after 48 h of treatment. In agreement with the results from the mRNA analysis, we found that RAW264.7 cells treated

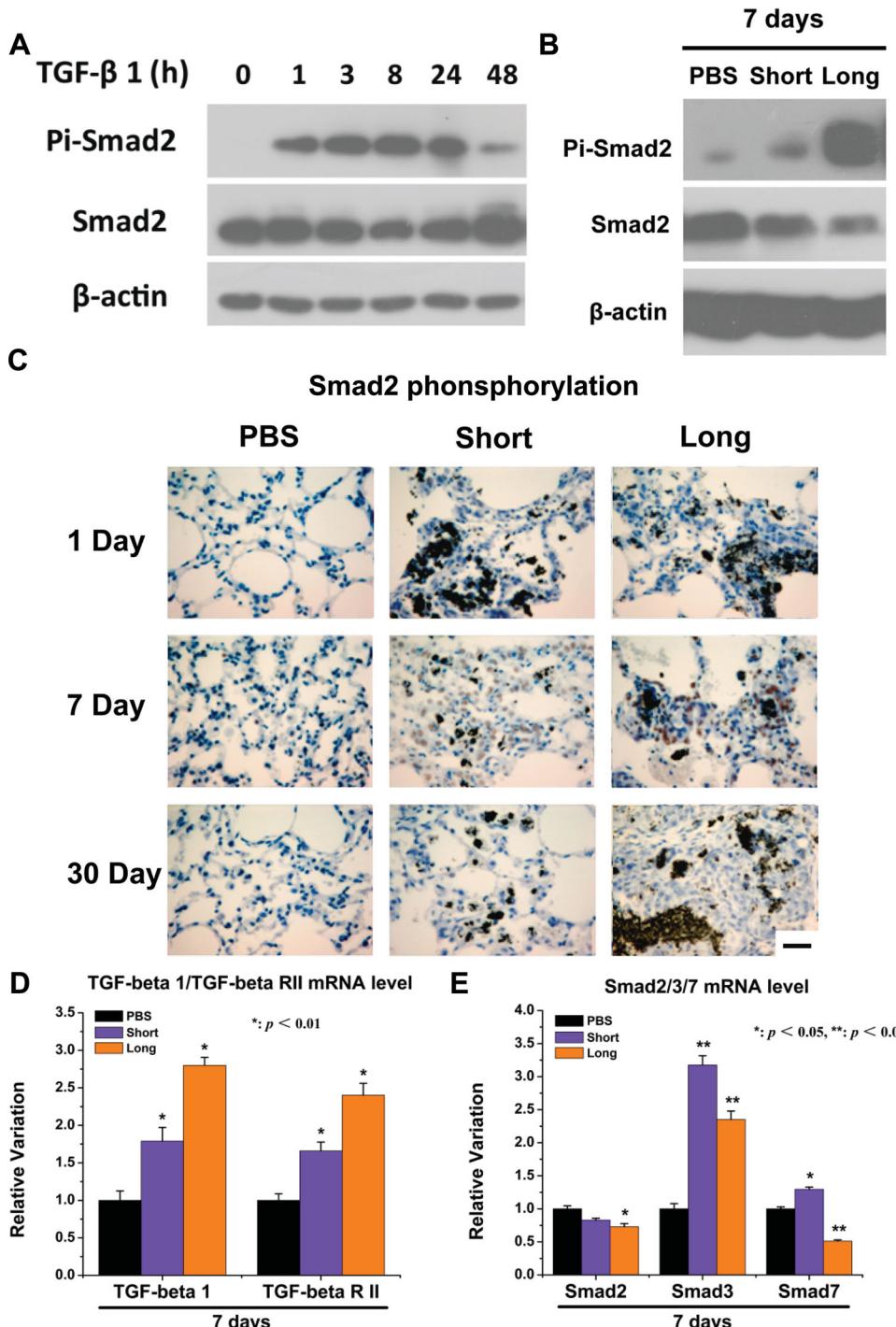


Figure 6. MWCNTs enhanced TGF- β 1-stimulated Smad2 phosphorylation in vivo. A) Immunoblotting was used to explore the time-course of Smad2 phosphorylation in NIH 3T3 cells, which were stimulated by 10 ng mL $^{-1}$ TGF- β 1 in vitro for the indicated time. B) SH rats were exposed by intratracheal instillation to short or long MWCNTs (0.6 mg/rat) for 1, 7 and 30 days. Immunoblotting was also used to examine the Smad2 phosphorylation level in lung tissues of SH rats exposed to short or long MWCNTs for 7 days. C) Immunohistochemistry was used to determine Smad2 phosphorylation in lung tissues of SH rats exposed to short or long MWCNTs for the indicated time. Scale bar 50 μ m. D,E) Real-time PCR was performed for detecting the relative mRNA expressions of D) TGF- β 1/T β RII and E) Smad2/3/7 at 7 days postexposure.

with long MWCNTs produced more TGF- β 1 and TNF- α than those treated with short MWCNTs (Figure 8C,D).

Finally, we co-cultured NIH 3T3 cells with RAW264.7 cells that had been pre-treated with a nontoxic concentration of MWCNTs for 24 h. After 24 h of co-culture, the mRNA

expression level of collagen III in NIH 3T3 fibroblast cells was detected by real-time PCR. As shown in Figure 8E, co-culture with MWCNT-treated RAW264.7 cells significantly increased the expression of collagen III in NIH/3T3 cells. Long MWCNTs caused a more significant upregulation of

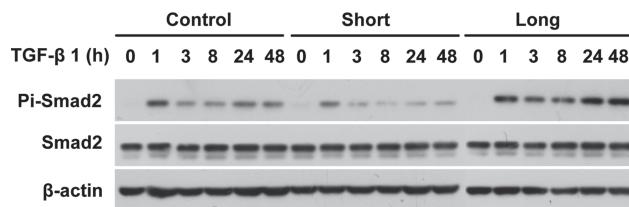


Figure 7. MWCNTs enhanced TGF- β 1-stimulated Smad2 phosphorylation in vitro. NIH 3T3 cells were first treated with short or long MWCNTs ($15 \mu\text{g mL}^{-1}$) for 24 h, and subsequently stimulated by 20 ng mL^{-1} TGF- β 1 for the indicated times. The time course of Smad2 phosphorylation was determined by immunoblotting.

collagen III than short MWCNTs. Moreover, we found that the addition of TGF- β 1 inhibitor into the co-culture medium significantly inhibited the effect of long MWCNTs on collagen III expression.

To further investigate the role of TGF- β 1 or TNF- α in upregulating collagen III expression, we used a specific antibody to neutralize RAW264.7-secreted TGF- β 1 or TNF- α . TGF- β 1 antibody inhibited the elevation of collagen III expression when it was added to the co-culture medium, but TNF- α antibody did not (Figure 8F). Together, these in vitro data suggest that TGF- β 1 secreted from MWCNT-stimulated RAW264.7 cells plays a central role in regulating collagen III expression.

Therefore, we propose the following framework for the cellular and molecular mechanisms of MWCNT-caused pulmonary fibrosis. As shown in **Figure 9**, MWCNTs first stimulate TGF- β 1 secretion in the lung by activation of alveolar macrophages and subsequently activate the TGF- β /Smad signaling pathway in fibroblasts, which can then upregulate the mRNA transcription of both ECM protease inhibitors and collagen type I/III. Moreover, our studies suggest that tube length acts as an important factor in MWCNT-induced pulmonary fibrosis.

3. Conclusion

It is crucial to systematically understand how these inherent and external factors influence the toxicity of carbon nanotubes so that their undesirable toxicity can be avoided. In summary, this study complements previous knowledge of the cellular and molecular mechanisms by which MWCNTs induce fibrosis, as well as the “length-dependent theory” used to explain CNT-caused harmful health effects. The fibrotic effect of MWCNTs clearly depends on the length of the MWCNTs. These data suggest that the activation of TGF- β /Smad2/collagen III signal transduction is an important step in the pulmonary fibrogenesis caused by long MWCNTs (Figure 9). Nevertheless, further investigation is still required to answer the question of whether MWCNT-activated TGF- β 1 production will further mediate the EMT process of epithelial cells in the lung. It will be interesting to find out whether MWCNTs could regulate TGF- β /Smad signaling through their interactions with TGF- β receptors. Furthermore, it will be useful to have identified the TGF- β /Smad2 signaling pathway as a source of potential and novel targets

that can be exploited for the development of therapeutic and preventive approaches to CNT-induced pulmonary fibrosis.

4. Experimental Section

Preparation and Characterization of MWCNTs: Two MWCNTs were purchased from Nanotech Port (Chengdu, China), which we called short or long MWCNTs based on their length. The length of short MWCNTs was $0.5\text{--}2 \mu\text{m}$, but for long MWCNTs, the length was $20\text{--}50 \mu\text{m}$. Their size and morphology was characterized by high-resolution transmission electron microscopy (HRTEM, Tecnai G2 F20 U-TWIN). Nanomaterial suspensions for experiments were prepared by weighing the materials into glass tubes and diluting them to 1.5 mg mL^{-1} stock solution with 1% Pluronic F108 solution in phosphate buffered saline (PBS), which was then sonicated for 1 h (500 W) at 30°C . For optimal suspension, the stock solutions were further sonicated for 30 min just before administration to cells. The suspensions of 1.5 mg mL^{-1} MWCNTs were further diluted into fresh medium to the final concentrations as required, and then old media were carefully removed and replaced with new media containing the final concentrations of MWCNTs.

Animals: Male spontaneously hypertensive (SH) rats (derived from Wistar Kyoto rats by segregation of the hypertensive trait and inbreeding), 11–12 weeks old and 220–250 g body weight, were obtained from Vital River Laboratory Animal Technology Co., Ltd, Beijing. Animals were housed as previously described.^[29] In our present study, SH rats were randomized by body weight into groups of PBS, short MWCNTs, and long MWCNTs (6 rats/group). SH rats were exposed to PBS or PBS-suspended short or long MWCNT particles (0.6 mg/rat) using a non-surgical intratracheal instillation once a day for two consecutive days. Then, the rats were killed 1 day, 7 days and 30 days following the last exposure. All animal studies, animal care, and use were approved according to local guidelines. All experiments with rats followed the guidelines for experimental animals and were approved by the animal welfare committee of Peking University.

Cell Culture and Co-Culture Experiments: The mouse leukemic monocyte macrophage cell line (RAW264.7) and mouse embryonic fibroblast cell line (NIH 3T3) were purchased from American Type Culture Collection (ATCC, Manassas, VA). RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum. NIH 3T3 cells were maintained in DMEM with 10% fetal bovine serum. Both media were supplemented with 2 mm L-glutamine, 100 U mL^{-1} penicillin and $100 \mu\text{g mL}^{-1}$ streptomycin. Cells were grown at 37°C in a humidified 95% air/5% CO_2 incubator. For co-culture experiments, we used 24 mm Transwell® with a $0.4 \mu\text{m}$ Pore Polyester Membrane Insert from Corning Company. RAW264.7 cells seeded in the well below were first treated with short or long MWCNTs ($15 \mu\text{g mL}^{-1}$) for 24 h, and then NIH 3T3 cells that had attached on the top of the insert for 24 h were co-cultured with RAW264.7 cells in the Transwell 6-well plate system for another 24 h.

Cell Viability Assay: Cell viability was determined using a cell counting kit-8 (Dojindo), in which 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) was used as a substrate. The relative number of surviving cells was determined in duplicate by estimating the

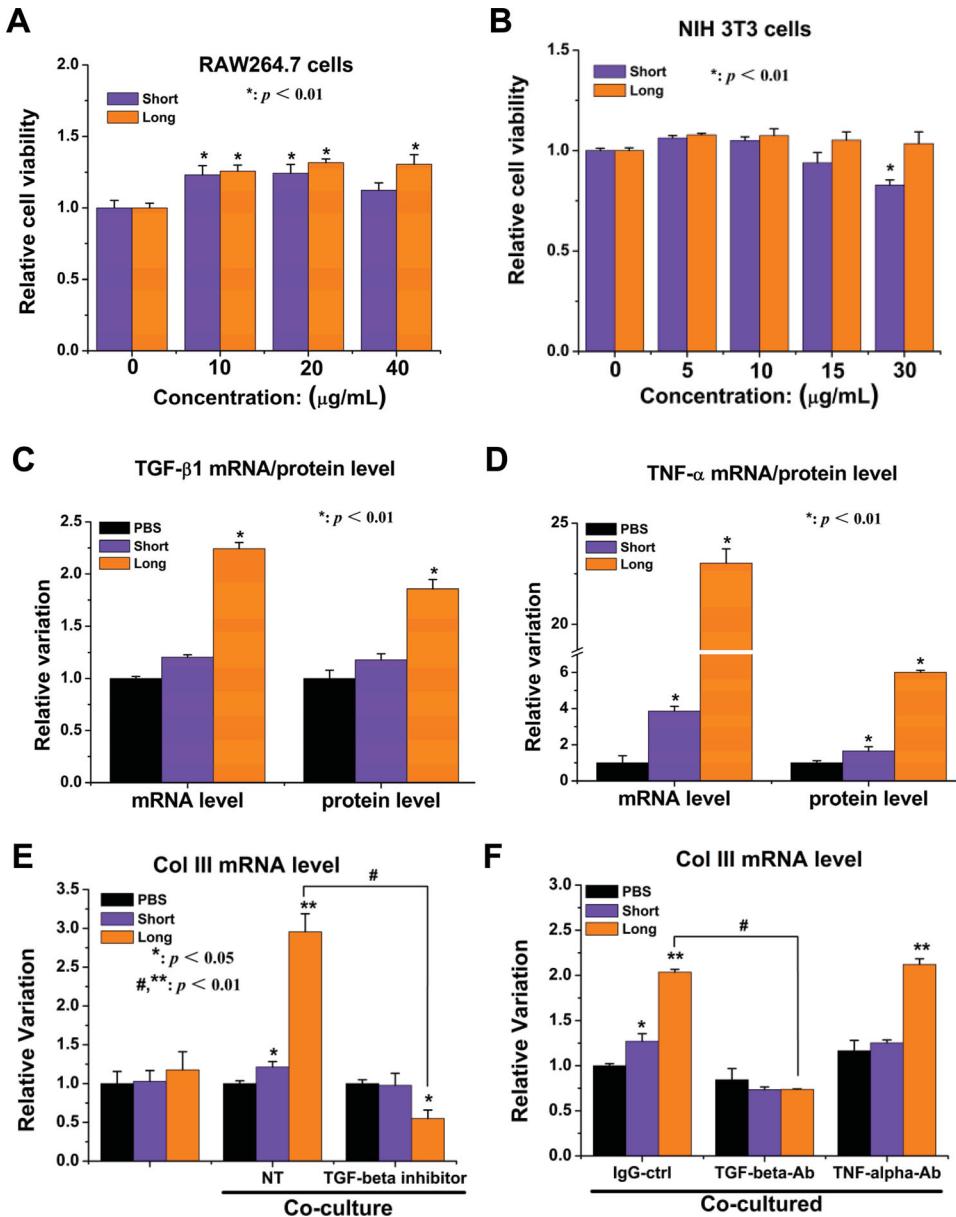


Figure 8. MWCNTs induce the production of collagen III in a TGF- β dependent manner. A,B) Effect of short or long MWCNTs on A) RAW264.7 and B) NIH 3T3 cell viability was detected by Cell Counting Kit-8 (CCK-8). All the CCK-8 values were normalized to the control (no MWCNT exposure), which represents 100% cell viability. C,D) RAW264.7 cells were treated with short or long MWCNTs ($15 \mu\text{g mL}^{-1}$). Real-time PCR was performed to determine the mRNA expression level of C) TGF- β 1 and D) TNF- α in RAW264.7 cells after 24 h of treatment. ELISA analysis was used to detect the concentrations of C) TGF- β 1 and D) TNF- α secreted by RAW264.7 after 48 h of treatment. All values were normalized according to the PBS control. E) In the left group, NIH 3T3 cells were directly exposed to short or long MWCNTs ($15 \mu\text{g mL}^{-1}$) for 24 h, without co-culture with RAW264.7 cells. In the middle and right groups, NIH 3T3 cells were co-cultured with RAW264.7 cells. Moreover, in the right group, a TGF- β 1 inhibitor was added to the co-culture medium, while there is no treatment (NT) for the middle group. For the co-culture experiment, RAW264.7 cells were treated with short or long MWCNTs ($15 \mu\text{g mL}^{-1}$) for the first 24 h, respectively, and then NIH 3T3 cells that had been attached for 24 h were put together with RAW264.7 cells. After another 24 h co-culture, NIH 3T3 cells were collected and real-time PCR was performed to detect their mRNA level of collagen III. F) MWCNTs treatment, cell co-culture and cell collection were performed in the same way as in (A), but the antibody of TGF- β 1 or TNF- α was added to the co-culture medium, respectively (The IgG was used as a negative control). (*) $P < 0.05$ and (**) $P < 0.01$ comparing control to MWCNT-treated cells; (#) $P < 0.01$ for pair-wise comparisons as shown. All values are means \pm SD from three independent analyses.

value of uninfected cells as 100%. 2×10^4 RAW264.7 or NIH 3T3 cells were planted in 96-well plates and grown overnight, and then each kind of cell was exposed to short or long MWCNTs at the indicated concentrations for 24 h; the cells were then incubated with the CCK-8 reagent for 1 h. Afterwards, the cells were

centrifuged at 2000 g for 10 min, and 80 μL of the supernatant transferred to a new plate. The plate was read at 490 nm in a UV-Vis spectrometer. All the CCK-8 values were normalized to the control (no MWCNT exposure), which represents 100% cell viability.

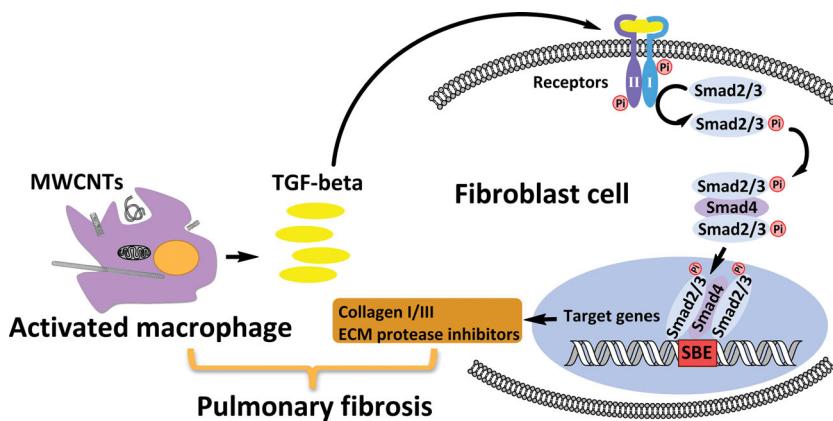


Figure 9. MWCNTs promote pulmonary fibrosis through the TGF- β /Smad signaling pathway.

Preparation of Bronchoalveolar Lavage Fluid: The right lung was used to obtain BAL fluid after ligation of the left bronchus. The right lung was lavaged three times with a specific volume (27 mL kg^{-1} body weight) of ice-cold phosphate-buffered saline (PBS, pH 7.4). The recovered BAL fluid was placed on ice. One aliquot of the recovered lavage fluid was centrifuged (400 g, 10 min, 4 °C), and then the supernatant fluid was collected and immediately analyzed for TGF- β 1 and TNF- α using the corresponding ELISA kits, and the pellets were collected for observation of MWCNT uptake in alveolar macrophages, which were stained with hematoxylin.

Histopathological and Immunohistochemistry Analysis: Unlavaged left lungs of each group were dissected and immediately fixed with 10% phosphate buffered formalin for 24–72 h. Lung tissues were then dehydrated, embedded in paraffin, and cut into 4 μm -thick slices. Sections were stained with hematoxylin and eosin (H&E) or Sirius Red to detect morphological changes and collagen deposition. For immunohistochemistry, slices were deparaffinized with xylene. Endogenous peroxidase was blocked with 0.3% H_2O_2 for 15 min. After treatment with blocking goat serum for 15 min, sections were incubated overnight with a collagen III antibody (1:200), an FSP-1 antibody (1:200) and a phospho-Smad2 antibody (1:200) and then with a biotinylated-link secondary antibody and peroxidase-labeled streptavidin followed by a revelation step with diaminobenzidine (substrate of peroxidase) and counterstaining with Mayer's hematoxylin. Slices were analyzed under a microscope (Leica DM4000M, Germany).

Real-Time Quantitative PCR Analysis: Total RNA was isolated from cells or lung tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) and converted to cDNA by M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. The resulting cDNA samples were analyzed by quantitative real-time PCR (Eppendorf, Hauppauge, New York, USA), using the SYBR Green assay. Relative levels of mRNA expression were normalized to β -actin expression for each gene. The following genes were measured: TNF- α , TGF- β 1, TGF- β receptor II, Smad2, Smad3, Smad 7, TIMP-1, PAI-1, Col I, Col III, and β -actin.

ELISA and Immunoblotting: TGF- β 1 and TNF- α levels in BALF or cell culture medium were quantified using DuoSet ELISA kits (R&D Systems, Minneapolis, MN) in accordance with the manufacturer's instructions. For immunoblotting, the cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 8.0,

150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 1 mM NaVO_4 , 10 mM NaF, and protease inhibitors), and the protein concentration in the lysates was determined by a spectrophotometer. Equal amounts of the lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and the immunoblotting was performed with various primary antibodies and secondary antibodies conjugated to horseradish peroxidase. Proteins were visualized by chemiluminescence.

Statistical Analysis: Data are represented as mean and SEM. Statistical analysis of data was carried out using Student's *t* test or the χ^2 test. A *P* value of less than 0.05 was considered to be a significant difference.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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