Sirtuin 6 inhibits MWCNTs-induced epithelial-mesenchymal transition in human bronchial epithelial cells via inactivating TGF-β1/Smad2 signaling pathway

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ABSTRACT

Multi-walled carbon nanotubes (MWCNTs) have been developed with numerous beneficial applications. However, rodent models demonstrate that exposure to MWCNTs via respiratory pathways results in pulmonary fibrosis. Therefore, they could elicit a potential risk of pulmonary fibrosis in humans due to occupational or consumer exposure. Sirtuin 6 (SIRT6), a nicotinamide adenine dinucleotide (NAD)-dependent deacetylase, has been proved to prevent fibrosis in the liver, renal and myocardial tissues. In this present study, we aimed to explore the role of SIRT6 in MWCNTs-induced epithelial-mesenchymal transition (EMT), one of the major contributor of lung fibrogenesis in human bronchial epithelial BEAS-2B cells. We found that the protein level of SIRT6 was elevated after exposure to MWCNTs in BEAS-2B cells. Overexpression of SIRT6 significantly inhibited MWCNTs-induced EMT and EMT-like cell behaviors in BEAS-2B cells. Moreover, wild-type SIRT6 was found to decrease MWCNTs-induced phosphorylation of Smad2, but not mutant SIRT6 (H133Y) without histone deacetylase activity. In conclusion, our study demonstrated that SIRT6 inhibited MWCNTs-induced EMT in BEAS-2B cells through TGF-β1/Smad2 signaling pathway, which depended on its deacetylase activity, and provided evidences that targeting SIRT6 could be a potential novel therapeutic strategy for MWCNTs-induced pulmonary fibrosis.

1. Introduction

Multi-walled carbon nanotubes (MWCNTs), made of multiple sheets of graphene arranged concentrically with diameters between 10 and 150 nm, have been developed with a wide range of applications in electrical engineering, aerospace industry and biomedicine, owing to their perfect mechanical strength, thermal conductivity, electrical and optical properties (ISO, 2008; Zhang et al., 2013). However, the rapid increase in the production and use of MWCNTs may lead to greater exposure of workers, consumers, and environment, which has raised considerable concerns over their potential effects on human health (Dong and Ma, 2015). In vivo studies have suggested that MWCNTs can induce pulmonary fibrosis in rats and mice when exposed via respiratory pathways (Muller et al., 2005; Porter et al., 2010; Mercer et al., 2011; Chen et al., 2014). Several researches suggest that MWCNTs exposure has a potential risk to induce pulmonary fibrosis in humans (Fatkhutdinova et al., 2016; Vlaanderen et al., 2017; Dahm et al., 2018), although it has not been confirmed by comprehensive epidemiologic studies. Pulmonary fibrosis is characterized as secreting excessive extracellular matrix (ECM), mainly collagens, resulting in scarring and destruction of the lung architecture, thereby reducing the exchange of oxygen and carbon dioxide (King Jr et al., 2011). Patients with pulmonary fibrosis have a poor prognosis, which may eventually lead to heart or lung failure and even death, causing great harm to human health and life (Evans et al., 2016). However, the treatment of pulmonary fibrosis is still a major problem that has not been completely solved by the medical community.

Sirtuin 6 (SIRT6) belongs to a family of nicotinamide adenine dinucleotide (NAD)-dependent deacetylases called sirtuins. It is located mainly in the nucleus, and considered to play a leading role in regulating genomic stability, cellular metabolism, stress response and aging (Jia et al., 2012). Recent studies have revealed that SIRT6 is of prime importance in protecting different organs from fibrosis. SIRT6 whole-body knockout mice exhibited increased interstitial fibrosis in...
hearts (Sundaresan et al., 2012). In addition, SIRT6 was found to inhibit cardiac fibroblasts differentiation into myofibroblasts via inactivation of nuclear factor xB signaling pathways (Tian et al., 2015). SIRT6 deficiency in the immune cells of mice resulted in liver fibrosis through activating c-JUN signaling (Xiao et al., 2012). The older SIRT6 knockout mice appeared enhanced renal fibrosis (Huang et al., 2017). We have previously found that SIRT6 was sufficient to restore TGF-β1-induced epithelial-mesenchymal transition (EMT) phenotype in human lung adenocarcinoma A549 cells (Tian et al., 2017), and overexpression of SIRT6 inhibited TGF-β1-induced myofibroblast differentiation in human fetal lung fibroblasts (HFL1) (Zhang et al., 2019). However, to the best of our knowledge, there was no report on the role of SIRT6 in MWCNTs-induced pulmonary fibrosis.

EMT is one of the key processes that contribute to the development of lung fibrosis (Tanjore et al., 2009; Chapman, 2011; Bartis et al., 2014). It is characterized by multiple molecular and biochemical changes affecting cell phenotype and behavior, during which epithelial cells progressively lose their characteristics and acquire traits of mesenchymal cells (Li et al., 2016). MWCNTs can induce EMT in lung epithelial cells, from which some fibroblasts involved in pulmonary fibrosis are thought to originate. Chen et al. (2014) found that lung epithelial cells could serve as a novel source of fibroblasts in MWCNTs-induced pulmonary fibrosis through EMT, and approximately 20% of epithelial cells transdifferentiated to fibroblasts after intratracheal instillation of MWCNTs in mice at 56 days. A transition from epithelial cell morphology to a mesenchymal cell phenotype was found in bronchoscopy-derived human bronchial epithelial cells treated with low doses of MWCNTs for 7 days (Snyder et al., 2014). Long MWCNTs were found to interact directly with rat type II alveolar epithelial cells (RLE-6TN), resulting in its loss of E-cadherin, an epithelial specific cell adhesion molecule, and gain of fibronectin expression thereby inducing EMT (Wang et al., 2015). TGF-β1-mediated EMT in human bronchial epithelial BEAS-2B cells was triggered by rod-like MWCNTs through AKT/GSK-3β/SNAIL signaling pathway (Polimeni et al., 2016). Therefore, inhibition of EMT process may contribute to the development of fundamental treatments for MWCNTs-induced pulmonary fibrosis.

Hence, the aim of this study was to investigate if SIRT6 regulated MWCNTs-induced EMT process in human bronchial epithelial BEAS-2B cells, which has been shown to be more sensitive to MWCNTs than A549 cells (Mishra et al., 2012), and explore the underlying molecular mechanisms. Our results showed that SIRT6 overexpression attenuated EMT process in BEAS-2B cells exposed to MWCNTs by inactivation of TGF-β1/Smad2 signaling pathway, which depended on its deacetylase activity. Therefore, targeting SIRT6 activation may be a promising therapeutic strategy in preventing and treating MWCNTs-induced lung fibrosis.

2. Materials and methods

2.1. Characterization of MWCNTs

MWCNTs were purchased from TIME NANO (Chengdu, China). The size and morphology of MWCNTs were characterized by transmission electron microscopy (TEM, JEM-2100, JEOL, Japan) and scanning electron microscopy (SEM, Zeiss, Germany). Thermal gravimetric analysis (TGA, STA7300, HITACHI, Japan) was used to analyze the purity of MWCNTs. The air-dried tube sheets were tested on X-ray diffraction (XRD, RigakuCo, Japan) to identify the elemental composition on the surface of MWCNTs. The specific surface area (SSA) of MWCNTs was measured by Brunauer-Emmett-Teller (BET, Quantachrome, Florida, USA) method. The mean hydrodynamic size of MWCNTs dispersed in cell culture medium was evaluated by dynamic light scattering (DLS, Zetasizer Nano-ZS, Malvern, Worcestershire, UK).

2.2. Dispersion of MWCNTs

The suspensions for experiments were prepared by weighing MWCNTs powders into a tube and diluting them to 1 mg/mL stock solution with 6 mg/mL bovine serum albumin (BSA, Sigma-Aldrich, USA) in ultra-pure water (Purelab Ultra, Elga). The suspensions were immediately vortexed for 20 s and then sonicated for 30 min in an ice water sonicator bath. The stock solution was further diluted into fresh medium to the final concentrations of MWCNTs. The stock solution should be resorcinated within 30 min before later use.

2.3. Cell culture and infection

BEAS-2B cell line, which was a generous gift from Dr. Chuanshu Huang (New York University), were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Gibco, Thermo Fisher Scientific, Beijing, China) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies, New Zealand), and 1% penicillin/streptomycin (Solarbio, Beijing, China). Cells were incubated at 37 °C in a humidified atmosphere with 5% CO₂ and 95% air without reaching confluence. Cells were infected with an adenoviral vector encoding SIRT6 (ad-SIRT6) or SIRT6 shRNA (sh-SIRT6) provided by Vigene Bioscience (Jinan, China). Catalytic inactive SIRT6 (H133Y) was kindly given by prof. Depel Liu (Peking Union Medical College). Cells were infected for 24 h before treatment with MWCNTs at 4 μg/cm² for 72 h.

2.4. Cytotoxicity assessment

Cell Counting Kit-8 (CCK8) assay was used to examine cell cytotoxicity. An equivalent number of cells (7.0 × 10⁴/well) was flat bottomed in 96-well plate and allowed to adhere for 24 h at 37 °C. Then the cells were treated with 0.25, 1, 4 and 16 μg/cm² of MWCNTs. Untreated cells were taken as control. After 72 h, 10 μL of CCK-8 reagent was added into each well, and the 96-well plates were incubated at 37 °C for 4 h. The plates were then centrifuged at 2000 g for 10 min and 80 μL of the supernatant was transferred to a new plate (Wang et al., 2015). Afterwards, the absorbance at 450 nm was measured by a multi-detection microplate reader (Model: Synergy 2; BioTek Instruments Inc., Winooski, VT, USA).

2.5. Transmission electron microscopy

BEAS-2B cells were exposed to 4 μg/cm² of MWCNTs for 72 h. Harvested cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) for 2 h and then washed with 0.1 M PB for three times. After postfixation in 1% OsO₄ in phosphate buffered saline for 2 h, the cells were dehydrated in a graded ethanol series, treated with propylene oxide, and embedded in epon. Embedded cells were cut into ultrathin sections (50–80 nm), mounted on copper grids, and finally stained with uranyl acetate and lead citrate. Images were recorded using TEM (JEM-1400plus, JEOL, Japan), operating at 120 kV with a Veleta CCD camera (Olympus, Japan).

2.6. Western blot

BEAS-2B cells were lysed on ice with a radioimmunoprecipitation assay buffer (Multisciences Biotech Co., Ltd., Hangzhou, China) containing 1% phenylmethanesulfonyl fluoride (Beityome, Shanghai, China) and 1% phosphatase inhibitors (Cell Signaling Technology, Danvers, MA, USA) for 30 min. The lysates were centrifuged at 14,000g at 4 °C for 15 min to remove cell debris. Protein concentrations in the supernatant were determined using Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Equal amounts of the lysates (20 μg) were subjected to 10% sodium dodeyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was incubated with...
primary antibodies overnight at 4 °C and then appropriate horseradish peroxidase-coupled secondary antibodies for 1 h at room temperature. Primary antibodies against p-Smad2 (3104), Smad2 (5339), p-Smad3 (9520), Smad4 (9523), SIRT6 (12486), vimentin (5741) were purchased from Cell Signaling Technology (Danvers, MA, USA). α-smooth muscle actin (α-SMA, ab124964) antibody was obtained from Abcam (Cambridge, UK). Anti-E-cadherin antibody (sc-7870) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies against MMP-2 (10373–2-AP) and MMP-9 (10375–2-AP) were purchased from Proteintech (Wuhan, China). The protein bands were visualized using enhanced chemiluminescence reagent (Multisciences Biotech Co., Ltd.). The density of the bands was quantified using Quantity One software.

2.7. Real-time reverse transcription polymerase chain reaction

Total RNA was extracted from BEAS-2B cells with TRIzol (TaKaRa, Kyoto, Japan) and converted into cDNA using Reverse Transcription kit according to the manufacturer’s protocol (TaKaRa, Kyoto, Japan). The mRNA levels were measured with SYBR using real-time PCR system according to the manufacturer’s protocol (TaKaRa, Kyoto, Japan). The 26.7. Real-time reverse transcription polymerase chain reaction

Cell migration was tested by transwell chambers (Corning, Kennebunk, Maine, USA). After treatment, 5 × 10^5 cells were seeded into the upper well of the chamber in 200 μL DMEM medium without FBS. 700 μL DMEM medium containing 10% FBS was added to the lower well to stimulate transwell process. Following incubation for 24 h, the cells remaining on the upper well were removed while the bottom cells were fixed with 4% paraformaldehyde (Merck KGaA, Germany), and then stained with 0.1% crystal violet and photographed for image analysis. The number of stained cells were counted using Image J software, and an average number within five randomly chosen fields was obtained.

2.9. Statistical analysis

All data were presented as the means ± standard deviation (SD) from three independent experiments. The results were analyzed by one-way ANOVA and Tukey’s post-hoc test using SPSS version 18.0. Difference was considered statistically significant with P < .05 and statistically highly significant with P < .01.

3. Results

3.1. Characterisation of MWCNTs

Characterization of MWCNTs was listed in Table 1. According to observations of TEM and SEM (Fig. 1A and B, respectively), the outer diameter of MWCNTs was 8–15 nm, the inner diameter was 3–5 nm, and the length was 10–50 μm. The TGA results demonstrated that the purity of MWCNTs was >98% (Fig. 1C). As shown in Fig. 1D, XRD pattern displayed typical (002) and (100) diffraction lines of MWCNTs. Trace metal analysis revealed that Fe and Ni were two major metal contaminants with contents of 0.2% and 0.5%, respectively. The surface area was estimated to be 140.77 m^2/g by BET analysis.

As is known, MWCNTs is highly hydrophobic and the formation of agglomerates in aqueous solutions or biological fluids is inevitable. Thus, the dispersion status and the stability of MWCNTs in culture medium were measured by DLS. Agglomerates had a highest average hydrodynamic diameter of 356 nm at 72 h (Fig. 1E). Meanwhile, the polydispersion index (PDI) was 0.45 at 72 h, indicating a wide distribution of agglomerate size (Fig. 1F). However, no significant change in hydrodynamic diameter and PDI were observed from 0 to 72 h (Fig. 1E and F). The results suggested good stability of MWCNTs dispersed in culture medium for at least 72 h.

3.2. Cellular uptake of MWCNTs

To study if MWCNTs could be internalized by BEAS-2B cells, cell were treated with 4 μg/cm^2 of MWCNTs for 72 h, and photographed by TEM. Untreated cells are shown in Fig. 2A. As shown in Fig. 2B and C, MWCNTs could be internalized by BEAS-2B cells, and detected mostly as agglomerates inside cellular vesicles in the cytoplasm. No MWCNTs was found in the nucleus or mitochondria of treated cells.

3.3. MWCNTs induce EMT in BEAS-2B cells

CCK-8 assay was first performed to determine the doses of MWCNTs used in the following study. BEAS-2B cells were treated with 0.25, 1, 4 and 16 μg/cm^2 (corresponding to 1.2, 4.8, 19.2 and 76.8 μg/mL, respectively) of MWCNTs for 72 h. As shown in Fig. 3A, compared with control, the viability of BEAS-2B cells was significantly decreased after treatment with MWCNTs at 1, 4 and 16 μg/cm^2 for 72 h (P < .05 or P < .01). The viability of the cells exposed to 1, 4 μg/cm^2 of MWCNTs was still >80%, while 16 μg/cm^2 of MWCNTs caused almost 50% death of the cells. Therefore, we used the doses of 0.25, 1 and 4 μg/cm^2 in the following study.

Cells undergoing EMT are defined by loss of epithelial marker proteins, such as E-cadherin, concomitant with the acquisition of mesenchymal marker proteins, such as vimentin and α-SMA. BEAS-2B cells were treated with MWCNTs at the concentrations of 0.25, 1, 4 and 16 μg/cm^2 for 72 h, and the ability of MWCNTs to induce EMT was examined. As shown in Fig. 3B, significant loss of E-cadherin after treatment with 1 and 4 μg/cm^2 of MWCNTs (P < .01), and increased expression of vimentin and α-SMA in response to MWCNTs at 0.25, 1 and 4 μg/cm^2 were observed in a dose-dependent manner (P < .05 or P < .01). Meanwhile, to investigate whether SIRT6 is associated with EMT, expression of SIRT6 was evaluated as well. Compared with that of control, the protein levels of SIRT6 were elevated as doses of MWCNTs increased, and achieved the highest level after treatment with 4 μg/cm^2 of MWCNTs at 72 h (P < .05; Fig. 3B). Immunofluorescent staining displayed that endogenously induced SIRT6 located mainly in the nucleus (Supplementary Fig. 1A).

3.4. Overexpression of SIRT6 is sufficient to inhibit MWCNTs-induced EMT in BEAS-2B cells

To explore whether SIRT6 is a vital regulatory factor in MWCNTs-induced EMT process, we next overexpressed SIRT6 using an adenoviral vector encoding SIRT6, and treated BEAS-2B cells with 4 μg/cm^2 of MWCNTs for 72 h. SIRT6 expression was strongly upregulated after ad-SIRT6 infection (Fig. 4A). Immunofluorescent staining demonstrated that enforced expression of SIRT6 located mainly in the nucleus.
Infection with the empty vector ad-GFP had no effect on the expression of E-cadherin and α-SMA compared with normal BEAS-2B cells (Supplementary Fig. 2). MWCNTs-induced downregulation of the epithelial marker E-cadherin and upregulation of the mesenchymal markers vimentin and α-SMA, were all restored by SIRT6 overexpression ($P < .05$ or $P < .01$; Fig. 4A). SIRT6 was next knocked down with an adenoviral vector encoding SIRT6 shRNA to further verify its negative regulatory role in EMT. After infection, BEAS-2B cells were treated with 4 μg/cm$^2$ of MWCNTs for 72 h. As shown in Fig. 4B, SIRT6 was successfully knocked down. There was no significant change in the expression of E-cadherin and α-SMA between normal cells and the cells infected with the empty vector sh-GFP (Supplementary Fig. 2). Unexpectedly, loss of SIRT6 failed to impact EMT markers in MWCNTs-treated cells (Fig. 4B). In summary, gain but not loss of SIRT6 regulated MWCNTs-induced EMT process in BEAS-2B cells.

![Supplementary Fig. 1B](image1)

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![Fig. 1. Characterization of MWCNTs. Representative TEM (A) and SEM (B) images of MWCNTs. (C) TGA curve of MWCNTs. (D) XRD pattern of MWCNTs. Hydrodynamic size (E) and PDI (F) of 4 μg/cm$^2$ of MWCNTs dispersed in culture medium were analyzed at 0, 24, 48 and 72 h by DLS.](image2)

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![Fig. 2. Representative TEM images to show the cellular uptake of MWCNTs by BEAS-2B cells in vitro. (A) Untreated cells were used as control. (B) BEAS-2B cells were treated with 4 μg/cm$^2$ of MWCNTs for 72 h and then the cellular uptake of MWCNTs was observed by TEM. (C) is the magnification image of (B).](image3)

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Fig. 3. MWCNTs induce EMT in BEAS-2B cells. (A) Effect of MWCNTs on the viability of BEAS-2B cells. BEAS-2B cells were treated with MWCNTs at 0.25, 1, 4 and 16 μg/cm² for 72 h. Untreated cells were used as control. Cell viability was evaluated by Cell Counting Kit-8 assay. (B) BEAS-2B cells were exposed to MWCNTs at the concentrations of 0.25, 1 and 4 μg/cm² for 72 h. Unexposed cells were taken as control. E-cadherin, vimentin, α-SMA and SIRT6 protein levels were measured by Western blot. All data were presented as the means ± SD from three independent experiments in the histograms. *P < .05, **P < .01.

Fig. 4. Overexpression of SIRT6 reverses MWCNTs-induced EMT in BEAS-2B cells. (A) BEAS-2B cells were infected with ad-GFP or ad-SIRT6 and treated with 4 μg/cm² of MWCNTs for 72 h. (B) BEAS-2B cells were infected with sh-GFP or sh-SIRT6, and treated with 4 μg/cm² of MWCNTs for 72 h. The protein levels of E-cadherin, vimentin, α-SMA and SIRT6 were measured by Western blot. All data were presented as the means ± SD from three independent experiments in the histograms. *P < .05, **P < .01.
3.5. SIRT6 regulates MWCNTs-induced EMT-like cell behaviors

In addition to phenotypic change, epithelial cells also experience functional transition that are characterized by synthesis and secretion of a series of profibrotic factors such as TGF-β1 upon exposure to continuous fibrogenic factors, which in turn triggers EMT and lung fibrosis. Chen et al. (2014) demonstrated that MWCNTs-induced TGF-β1 served as autocrine signal to stimulate EMT-like alteration in A549 cells. Moreover, synthesis of matrix metalloproteinases (MMPs) may facilitate the migration of fibrocytes and also contribute to ECM remodeling during the development of idiopathic pulmonary fibrosis (IPF) (Pardo et al., 2016). Thus, we examined if SIRT6 could inhibit EMT by abrogating those fibrotic responses in cells exposed to MWCNTs. After infected with ad-SIRT6, BEAS-2B cells were treated with 4 μg/cm² of MWCNTs for 72 h. The mRNA levels of MMP-2 (A), MMP-9 (B), COL3A1 (C) and TGF-β1 (D) were measured by real-time PCR. The protein levels of MMP-2 and MMP-9 were measured by Western blot (E). After the indicated treatment, BEAS-2B cells were seeded in the transwell chamber. Following incubation for 24 h, the bottom cells were stained with 0.1% crystal violet and photographed for image analysis (magnification, 100×). The number of stained cells were counted using Image J software, and an average number within five randomly chosen fields was obtained. All data were presented as the means ± SD from three independent experiments in the histograms. *P < .05, **P < .01. (For interpretation of references to colour in this figure legend, the reader is referred to the web version of this article.)

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expression of these genes. However, the mRNA levels of MMP-2, MMP-9, TGF-β1 and COL3A1 and the protein levels of MMP-2 and MMP-9 were significantly reversed by overexpression of SIRT6 in response to MWCNTs (P < .05 or P < .01; Fig. 5A-E). In EMT process, epithelial cells acquire fibroblast-like properties and show reduced intercellular adhesion and increased motility. We found that forced expression of SIRT6 almost completely restored MWCNTs-induced migratory behavior (Fig. 5F). Altogether, these results suggested that SIRT6 could inhibit MWCNTs-induced fibroblast-like cell behaviors.

3.6. SIRT6 overexpression suppresses MWCNTs-induced Smad2 activation

TGF-β/Smad is a canonical pathway involved in EMT. It has been demonstrated that MWCNTs-induced EMT could be mediated by TGF-β1 and the resultant immediate-but transient-activation of Smad2 (Chen et al., 2014; Wang et al., 2015). To investigate if SIRT6 inhibited MWCNTs-induced EMT through TGF-β/Smad signaling pathway in BEAS-2B cells, phosphorylation of Smad2 and Smad3 was examined by overexpressing SIRT6 in the absence or presence of MWCNTs. As shown in Fig. 6A, compared with those of negative control ad-GFP, p-Smad2 and p-Smad3 were significantly upregulated after exposure to 4 μg/cm² of MWCNTs for 1 h (P < .01). SIRT6 overexpression significantly ameliorated phosphorylation of Smad2 (P < .01), but not Smad3 in BEAS-2B cells treated with MWCNTs. These results indicated that SIRT6 could ameliorate EMT via inhibiting MWCNTs-induced Smad2 activation.

Phosphorylated R-SMADs (Smad2 and Smad3) form complexes with the co-activator Smad4, then translocate into the nucleus where the
Smad4-containing complexes are directly involved in regulating the transcription of target genes, such as the ECM mRNAs, both positively and negatively (Schmierer and Hill, 2007). Therefore, the protein levels of Smad4 in BEAS-2B cells infected with SIRT6 after exposure to 4 μg/cm² of MWCNTs for 72 h were measured as well. Our results demonstrated that SIRT6 overexpression had no influence on the protein levels of Smad4 in the absence or presence of MWCNTs (Fig. 6A).

Since the histone deacetylase activity of SIRT6 plays a vital role under pathophysiological conditions, we next examined Smad2 phosphorylation in BEAS-2B cells infected with mutant SIRT6 (H133Y) without histone deacetylase activity when exposed to MWCNTs at 4 μg/cm² for 1 h. As shown in Fig. 6B, mutant SIRT6 failed to repress Smad2 phosphorylation induced by MWCNTs, suggesting that SIRT6 inhibited MWCNTs-induced Smad2 activation depending on its deacetylase activity in BEAS-2B cells.

4. Discussion

The potential of MWCNTs to induce fibrosis in lungs has been a major concern in both occupational and environmental settings. The fibrotic lesions induced by MWCNTs bear a high degree of similarity to pneumonia and IPF, both of which are human lung fibrotic diseases that are frequently progressive and incurable. Therefore, understanding of the mechanisms is necessary to promote prevention and treatment of MWCNTs-induced lung fibrosis. In this study, we found that SIRT6 was a key molecule in the regulation of MWCNTs-induced EMT in BEAS-2B cells. Overexpression of SIRT6 negatively regulated EMT process under the treatment of MWCNTs, and TGF-β1/Smad2 signaling pathway was involved in SIRT6-mediated inhibition of EMT, which relied on its deacetylase activity. Therefore, targeting SIRT6 activation could be possibly utilized to develop novel therapeutic approaches to prevent or treat MWCNTs-induced lung fibrosis.

The physicochemical properties of MWCNTs are of great importance in determining their reactive potential. Several studies addressed that pulmonary fibrosis depended on the length of MWCNTs. Long but not short MWCNTs were found to significantly increase fibrosis in the lungs of MWCNTs-exposed mice or rats (Wang et al., 2013; Chen et al., 2014). According to a recent study, there was an increase in fibrogenic activity of MWCNTs as the length of MWCNTs increased (Hindman and Ma, 2018). Therefore, we used long MWCNTs (10–50 μm) to promote EMT and examine the role of SIRT6 in MWCNTs-induced EMT. The aggregation and dispersal state of MWCNTs has been shown to impact the fibrogenic effects in epithelial cells and macrophages in vitro by changing the overall surface area available for interaction with target cells and molecules (Wang et al., 2011). We used submersed cell culture instead of aerosolized MWCNTs which better mimic real human exposure conditions in the present study. One major limitation of MWCNTs in a liquid dispersion is that MWCNTs probably undergo much more agglomeration and subsequently result in more sedimentation compared with aerosol exposure (Brandenberger et al., 2010). To prevent MWCNTs aggregation, we used BSA as a dispersant to allow efficient interaction with the cells. We observed no significant change in hydrodynamic diameter and PDI following treatment with MWCNTs up to 72 h, indicating existence of a stable suspension during incubation. It should be noted that hydrodynamic diameters for MWCNTs are defined as the equivalent spherical diameters (i.e., the diameter of a sphere with the same translational diffusion coefficient) and cannot be simply related to the exact particle sizes. Therefore, the hydrodynamic diameters of MWCNTs measured here only represent their relative sizes. Moreover, other parameters such as SSA, purity and metal contents, have been shown to influence their reactivity as well (Shvedova et al., 2009). In our study, we selected high-purity MWCNTs with low contents of Fe (0.2%) and Ni (0.5%), which were supposed to have little or no influence on MWCNTs-induced EMT or lung fibrosis (Jr et al., 2012; Polimeni et al., 2016). As suggested by Vietti et al. (2013), the in vivo mouse doses (12.5–100 μg/mouse) of MWCNTs is roughly equivalent to 25–200 mg/lung in humans, which could be reached after 10 months to 6.2 years of human inhalation exposure, where MWCNTs air borne level was 40 μg/m³ and ventilation was 20 L/min. Mice exposed to 12.5–100 μg/lung (alveolar surface area 0.05 m²) is equivalent to 0.025–0.2 μg/cm² in cell culture. However, considering much shorter exposure time in vitro than in vivo, irregular MWCNTs distribution in lung and <100% MWCNTs deposition in vitro, a higher doses of MWCNTs (7.5–30 μg/cm²) was used in fibroblast study. In our study, the results of CCK-8 assay showed that 16 μg/cm² of MWCNTs caused almost 50% death of BEAS-2B cells. We therefore selected 4 μg/cm² as the highest dose, which caused <20% cell death. What’s more, the doses of 5.5–44 μg/mL (corresponding to 1–8 μg/cm²) have been used to study MWCNTs-induced EMT in BEAS-2B cells by Polimeni et al. (2016). Therefore, 0.25–4 μg/cm² of MWCNTs used in our study is considered to be relevant with human exposure scenarios and also comparable to those of other study.

Our experiments demonstrated that MWCNTs induced SIRT6 expression while SIRT6 overexpression inhibited MWCNTs-induced EMT in BEAS-2B cells. SIRT6 is located mainly in the nucleus (Jia et al., 2012). Our immunofluorescent results also demonstrated that both endogenously induced SIRT6 and enforced expression of SIRT6 located mainly in the nucleus. Therefore, the observation that MWCNTs induced SIRT6 expression while overexpression inhibited MWCNTs-induced EMT in BEAS-2B cells is not due to differential cellular localization of endogenously induced SIRT6 and enforced expression of SIRT6. We speculated that upregulation of SIRT6 might be an insufficient compensatory mechanism in response to MWCNTs. However, the increase of endogenous SIRT6 is not enough to inhibit MWCNTs-induced EMT. The phenomenon that a significant upregulation of SIRT6 was also reported in response to TGF-β1, paracortat, H₂O₂, and angiogenins II (Ang II). Under TGF-β1 stimulation, SIRT6 expression was increased at the mRNA and protein levels in a dose-dependent manner in human bronchial epithelial cells isolated from IPF lung tissue, and overexpression of SIRT6 inhibited TGF-β1-induced cellular senescence in human bronchial epithelial cells (Minagawa et al., 2011). Endogenous SIRT6 levels was elevated in human fibroblasts subjected to paracortat or H₂O₂ treatments, and overexpression of SIRT6 was found to promote DNA repair under oxidative stress induced by paracortat or H₂O₂ (Zhiyong et al., 2011). In Ang II-induced hypertrophic cardiomyocytes and abdominal aortic constriction-induced hypertrophic hearts of rats, the expression of SIRT6 protein was upregulated, and overexpression of SIRT6 could attenuate Ang II-induced cardiomyocyte hypertrophy (Yu et al., 2013). Taken together, these studies suggested that SIRT6 was upregulated in response to different stimuli, possibly as an insufficient compensatory mechanism, and SIRT6 overexpression could play a protective role in response to these stimuli, which were consistent with our findings.

EMT has been implicated as a vital process leading to the occurrence of lung fibrosis (Tanjore et al., 2009; Chapman, 2011; Barts et al., 2014). Therefore, reverse or inhibition of EMT progress is a feasible therapeutic approach. To the best of our knowledge, we first reported that SIRT6 overexpression was able to attenuate MWCNTs-mediated EMT, as well as EMT-like cell behaviors, which implicated that proper regulation of the cellular plasticity by SIRT6 might be an effective therapeutic method for MWCNTs-induced EMT. However, our result displayed that loss of SIRT6 had no effect on MWCNTs-induced EMT, this phenomenon may be attributed to that there are seven members in sirtuin family, and once SIRT6 is knocked down, other member may compensate for the loss of SIRT6 function (Frye, 2000). For example, recent study suggested that SIRT1 could antagonize the invasion of ovarian cancer cells by suppressing EMT process (Ray et al., 2017).

We further explored the possible mechanisms by which SIRT6 suppresses MWCNTs-induced EMT. It has been reported that Smad2 was activated in MWCNTs-induced EMT in A549 and RLE-6TN cells (Chen et al., 2014; Wang et al., 2015). Moreover, activation of Smad2 or both of Smad2 and Smad3 by MWCNTs resulted in increased lung
fibrosis in rats or C57BL/6 J mice, respectively (Wang et al., 2013; Dong and Ma, 2017). Therefore, we investigated whether Sirt6 modulated MWCNTs-induced EMT process by inhibiting TGF-β1/Smad2 signaling pathway. Our results revealed that Sirt6 prevents MWCNTs-induced EMT process by inhibiting TGF-β1/Smad2 signaling pathway. However, how SIRT6 modulates phosphorylation of Smad2 is still unknown. Here, we speculate that over-expression of Sirt6 inhi...
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