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Article

Multi-walled carbon nanotubes (MWCNTs) cause cellular senescence in TGF-β stimulated lung epithelial cells

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Abstract: Multi-walled carbon nanotubes are engineered nanomaterials (ENMs) that have a fiber-like structure which may be a concern for the development of cellular senescence. Premature senescence, a state of irreversible cell cycle arrest, is implicated in the pathogenesis of chronic lung diseases such as pulmonary fibrosis (PF). However, the crosstalk between downstream pathways mediating fibrotic and senescent responses of MWCNTs are not well defined. Here, we exposed human bronchial epithelial cells (BEAS2B) to MWCNTs for up to 72 hours and demonstrate that MWCNTs increase reactive oxygen species (ROS) production accompanied by inhibition of cell proliferation. In addition, MWCNTs exposure resulted in the increase of p21 protein abundance and senescence associated β-galactosidase (SA β-gal) activity. We also determined that co-exposure with the cytokine, transforming growth factor-β (TGF-β) exacerbated cellular senescence indicated by increased protein levels of p21, p16, and γH2A.X Furthermore, the production of fibronectin and plasminogen activator inhibitor (PAI-1) was significantly elevated with the co-exposure compared to MWCNTs or TGF-β alone. Together, our study suggests that the senescence potential of MWCNTs may be enhanced by pro-fibrotic mediators in the surrounding microenvironment.

Keywords: MWCNT; senescence; pulmonary fibrosis, epithelial

1. Introduction

Multi-walled carbon nanotubes (MWCNTs) are fiber-like engineered nanomaterials (ENMs) with unique physiochemical properties that have promising applications in a variety of biomedical applications including tissue scaffolding, drug delivery systems, and biosensors [1]. Production of MWCNTs has risen rapidly over the past decade, prompting public health concerns regarding their possible adverse health effects [2]. One of the main concerns with exposure to fiber-like ENMs is pulmonary fibrosis (PF). PF is a progressive interstitial lung disease that arises from excessive deposition and production of extracellular matrix (ECM) proteins by activated myofibroblasts. Due to their small diameter (<100 nm), MWCNTs can penetrate into the deeper regions of the lung. In addition, their chemical stability and high aspect ratio impedes phagocytosis and clearance by lung macrophages, resulting in biopersistence[3]. In rodent studies, inhalation of MWCNTs triggers airway inflammation and can potentiate fibrotic responses [4], implicating MWCNTs in the development of PF and other chronic lung diseases. However, the mechanisms underlying the MWCNT induced fibrotic response is not fully understood.

Cellular senescence, a state of irreversible growth arrest, is implicated in both aging and the development of age-associated chronic lung diseases such as PF. Analysis of lung tissues from patients show increased levels of established senescence biomarkers such as p21, p16, and senescence associated β-galactosidase (SA β-gal) [5, 6]. Premature senescence can be induced by a variety of pro-aging stressors including redox imbalance, telomere shortening, and genomic instability [6]. Moreover, the adoption of a secretome known as the senescent associated secretory phenotype (SASP) results in the release of a collection of cytokines, growth factors, and matrix metalloproteinases that drive

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pathologic lung remodeling and inflammation [7]. The involvement of senescence in PF is further supported by the use of senolytic drugs [8]. The clearance of senescence cells has been shown to improve pulmonary function and inhibit the fibrotic secrotome in rodents *in vivo* [6, 9]. A clinical trial also demonstrated that administration of senolytics to PF patients moderately improvement patient clinical scores in mobility assessments [10]. Together, these studies demonstrate a clear role for senescence in the development of PF.

Many reports indicate that exposure to MWCNTs can lead to the development of pulmonary fibrosis *in vivo* [4, 11, 12] and drive pro-fibrotic epithelial-mesenchymal transition (EMT), and myofibroblast differentiation and activation *in vitro* [13, 14]. Previous studies also indicate that certain MWCNTs can result in increased oxidative stress and DNA damage to lung epithelial cells, which may lead to cellular senescence [15, 16]. However, the role of cellular senescence in MWCNT induced fibrotic responses is poorly understood. Thus, we hypothesized that MWCNT would cause cellular senescence that would be exacerbated under pro-fibrotic conditions. We show that MWCNT exposure modestly increases senescence biomarkers under normal conditions. We further show that MWCNTs exacerbated the cellular senescence induced by TGF-β. Together, our results suggest that the overlap between pro-fibrotic and pro-senescent pathways mediates the adverse effects of MWCNT exposure.

2. Materials and Methods

Cell Culture and Treatment

Human bronchial epithelial cells (BEAS-2B, ATCC, VA) were cultured in DMEM-Ham's F12 50:50 mixture (Cat#: 1130032 Thermo Fischer Scientific Waltham, MA) supplemented with 5% FBS (Cat#: 10438026, Thermo Fisher Scientific, Waltham, MA), 15 mM HEPES, penicillin (100 U/mL), and streptomycin (100 μg/mL). Cells were cultured to 80% confluency and serum deprived overnight before exposure. MWCNTs (< 8 nm diameter, 10-30 μm length, https://www.cheaptubes.com) were suspended in dispersion media (0.6 mg/mL BSA, 5.5mM D-glucose, and 0.01mg/mL dipalmitoylphosphatidylcholine in PBS) and probe sonicated for 10 minutes to create a stock solution (2mg/mL). MWCNT were sonicated before each treatment for 5 minutes and diluted in serum free culture media to desired concentrations for exposures. Dispersion media was diluted in culture media for vehicle controls and had no effect on any measured parameters.

Cell Viability and ELISA

For cell viability measurements, cells were treated with MWCNTs for 24 hours, then cells were detached by 0.25% trypsin and neutralized with complete medium. Cells were mixed with equal volume of ViastainTM AO/PI (Nexelcom Biosciences) and counted on a Cellometer Auto 2000. Absolute cell count and viability were used to determine cytotoxicity. For cell proliferation measurements, cells were grown to 50% confluency and treated for 48 hours and then counted as described above. IL-8 was measured in cell supernatants using an Invitrogen IL-8 detection kit (Cat#: CHC1303, Thermo Fisher Scientific, Waltham, MA) and done according to the manufacturers' instructions.

Cellular Reactive Oxygen Species (ROS) Production

Cellular ROS production was determined using 2', 7'dichlorofluorescein diacetate (DCF-DA) fluorogenic probe (EMD Bioscience, CA). Briefly, serum deprived cells were incubated with 5 mM DCF-DA reagent for 45 minutes at 37°C. Then the media was aspirated and replaced with MWCNT treatments. Non-DCFDA treated cells were used for blank subtraction (ΔF) and the data represented as fold change over controls. Fluorescence intensities of cells were measured by Cytation 5 Cell Imaging Multi-Mode Reader (BioTek Instruments, Inc., VT, US)

Western Blotting

Protein concentrations were measured in whole cell lysates by Pierce BCA Protein Assay Kit (Cat#: 23225, Thermo Scientific). Total 20μg protein was separated in gradient SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was incubated with anti-Fn (ab2413 1:1000, Abcam), anti-PAI-1(ab182973, 1:1000, Abcam), anti-p21 (ab109199 1:1000, Abcam), anti-p16 (ab211542 1:1000, Abcam), anti-γH2A.X (ab2893, 1:1000, Abcam), anti-H2A.X (ab11175, 1:1000, Abcam), anti-E-cadherin (ab11472 1:1000, Abcam), and anti-vimentin (ab92547 1:1000, Abcam) primary antibodies overnight at 4°C. Membranes were incubated with HRP-conjugated secondary anti-rabbit

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(Cat#1706515, 1:5000, BioRad), or anti-mouse (Cat#: 1706516 1:5000, BioRad) for 1 hr at room temperature. The chemiluminescence was detected using the Bio-Rad ChemiDoc MP imaging system. Densitometric analyses of the band intensities were performed using Image Lab software (v4.1, BioRad, Hercules, CA).

Cellular Senescence Activity Assay

Detection of SA β-gal activity was determined by using a Cellular Senescence Activity Assay (Cat#: ENZ-KIT129, Enzo Life Sciences, Farmingdale, NY). The assay was performed according to the manufacturer's instructions. Senescence activity was defined as the fluorescence intensity at 360/465nm.

Statistical Analysis

Statistical analyses of significance were performed by one-way ANOVA followed by Tukey's multiple comparison test when comparing multiple groups using GraphPad Prism 7 (La Jolla, CA). Data are presented as means ± SEM. $p < 0.05$ is considered as statistically significant.

3. Results

Cellular responses to MWCNTs in human bronchial epithelial cells

MWCNTs were evaluated for their cytotoxicity and potential to induce inflammatory and oxidative stress responses. BEAS2B cells were exposed to MWCNTs for 24 hours and then subsequently stained with AO/PI. Cell counts did not change with treatments and displayed no cytotoxicity up to 50 μg/mL, (Fig 1A). In addition, ROS was significantly elevated at 8 hours (Fig 1B). Levels of IL-8 trended upwards with increasing MWCNT dose, but were not significantly different (Fig 1C). In cell proliferation assays, MWCNTs led to a dose dependent decrease in cell counts after 48 hours at concentrations higher than 0.5 μg/mL (Fig 1D and 1E).

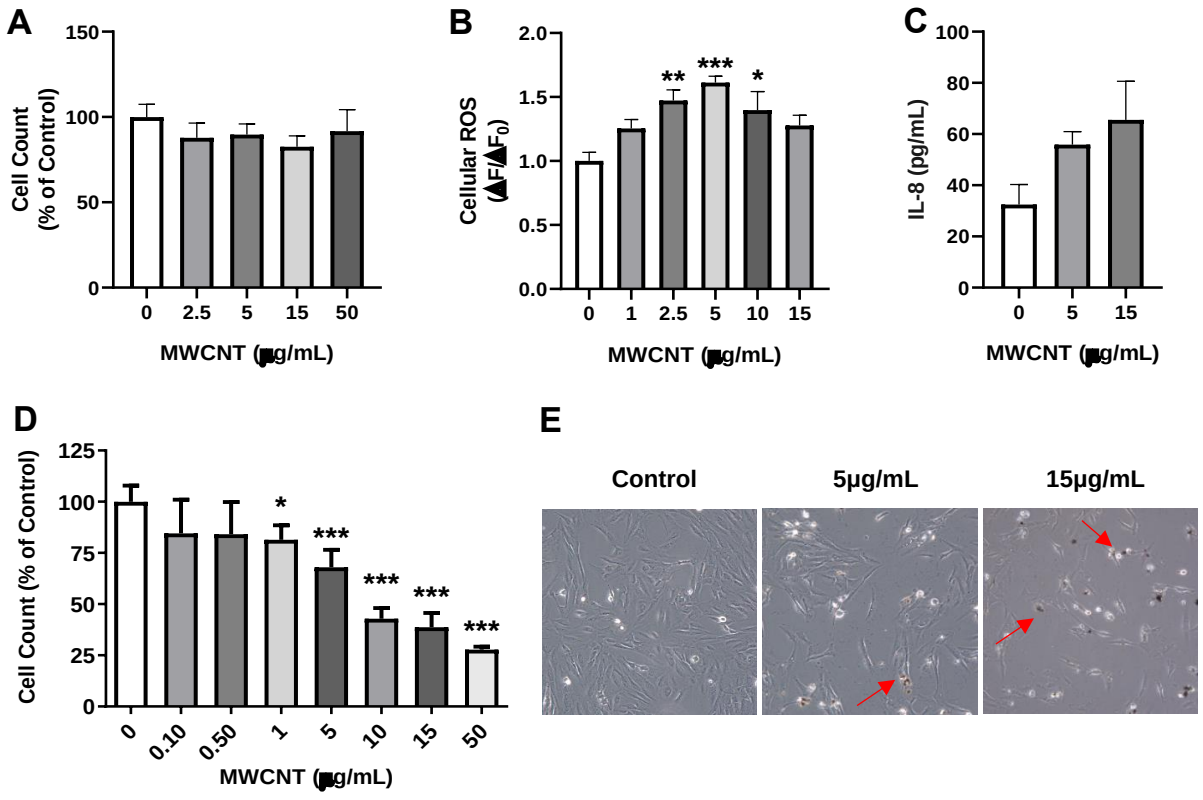

Figure 1: Cellular responses to MWCNT in BEAS2B. (A) Cytotoxicity was evaluated by total cell counts 24 h post exposure. Cells were rinsed and stained with AO/PI and then counted in a Cellometer 2000 (N=6). (B) Cellular ROS was determined using DCFDA 8 h post exposure (N=6). (C) IL-8 release was assessed by ELISA 24 h post exposure (N=3). (D) Cell growth was assessed by AO/PI staining 48 h post-exposure. (E) Light microscopy shows MWCNTs are

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found in dead cells indicated by the red arrows. Data are expressed as mean ± SEM (N ≥ 3). Statistical significance was determined by one-way ANOVA (Tukey's multiple comparison test). *p < 0.05, **p < 0.01, ***p<0.001 indicates significance.

MWCNT and TGF- β exposure propagate cellular senescence

BEAS2B cells were treated with MWCNTs and/or TGF-β for 72 hours, then evaluated for the markers of cellular senescence and cell cycle arrest. MWCNT exposure led to elevated protein levels of SA β-gal, and p21; a non-significant increased protein abundance of p16, γH2A.X, and H2A.X was also observed after MWCNT treatment. Co-treatment (MWCNT with TGF-β) resulted in elevated levels of p21, p16, and γH2A.X compared to either TGF-β or MWCNT alone (Fig 2). The full blots are shown in the appendix (Fig S1)

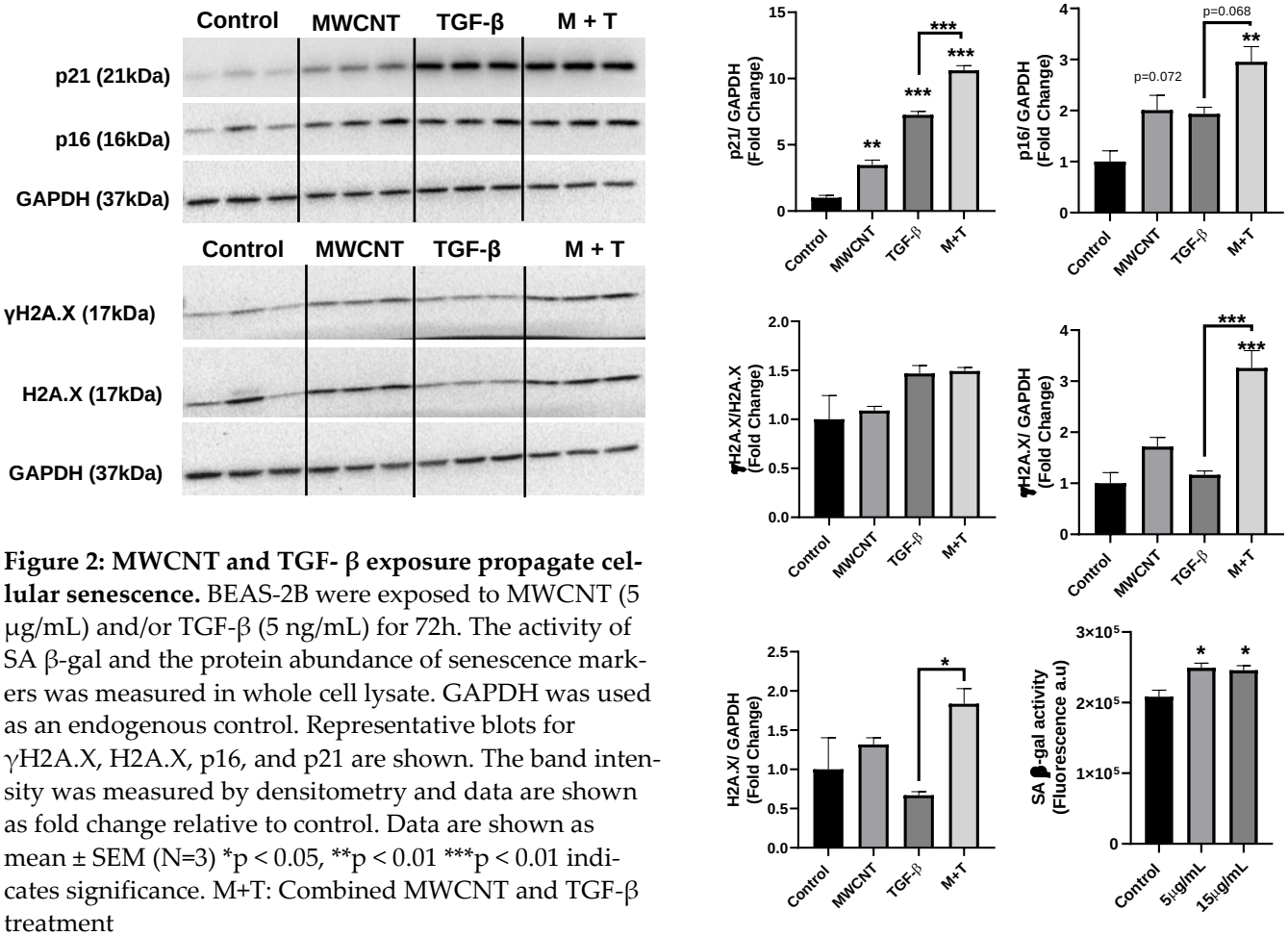

Figure 2: MWCNT and TGF- β exposure propagate cellular senescence. BEAS-2B were exposed to MWCNT (5 μg/mL) and/or TGF-β (5 ng/mL) for 72h. The activity of SA β-gal and the protein abundance of senescence markers was measured in whole cell lysate. GAPDH was used as an endogenous control. Representative blots for γH2A.X, H2A.X, p16, and p21 are shown. The band intensity was measured by densitometry and data are shown as fold change relative to control. Data are shown as mean ± SEM (N=3) *p < 0.05, **p < 0.01 ***p < 0.01 indicates significance. M+T: Combined MWCNT and TGF-β treatment

MWCNT and TGF- β induce EMT and increase ECM production

BEAS2B cells were treated with MWCNTs and/or TGF-β for 72 hours and evaluated for the production of EMT and ECM proteins. MWCNT exposure resulted in decreased E-cadherin protein levels (Fig 3). TGF-β alone or TGF-β + MWCNT treatment dramatically decreased the protein levels of E-cadherin while there is no difference in between the two treatments (Fig 3). For mesenchymal marker measurement, co-treatment of MWCNTs with TGF-β led to an increased protein abundance of PAI-1 and fibronectin compared to TGF-β alone (Fig 3). There is no significant difference of protein levels of vimentin among control, MWCNT and TGF-β group; while the co-treatment of MWCNT with TGF-β significantly up-regulated the protein abundance of vimentin compared control (Fig 3). The full blots are shown in the appendix (Fig S2)

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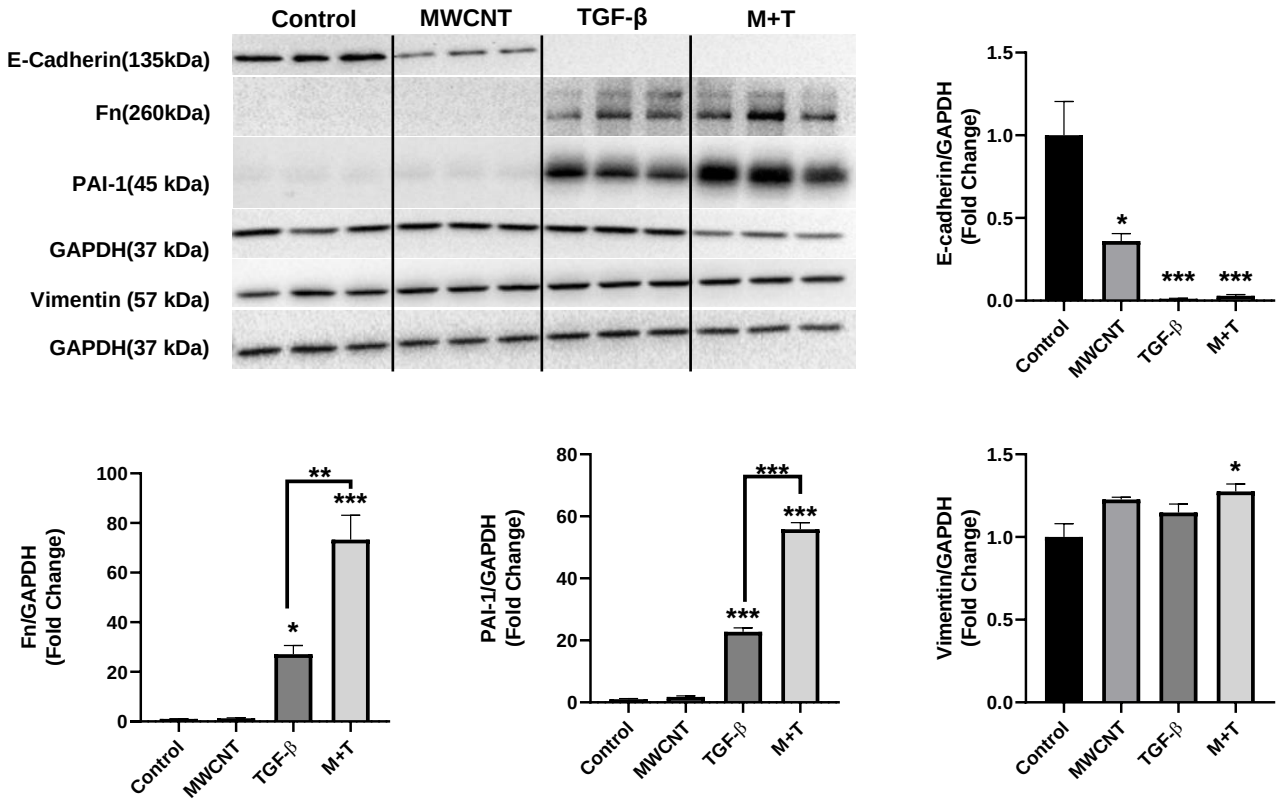

Figure 3: MWCNT and TGF- β induce EMT and increase ECM production. BEAS-2B were exposed to MWCNT (5 μg/mL) and/or TGF-β (5 ng/mL) for 72h. The protein abundance of ECM related markers was measured in whole cell lysate using western blotting. GAPDH was used as an endogenous control. Representative blots for E-cadherin, vimentin, fibronectin (Fn), and plasminogen activator inhibitor-1 (PAI-1) in BEAS-2B are shown. The band intensity was measured by densitometry and data are shown as fold change relative to control; Data are shown as mean ± SEM (n = 3/group) *p < 0.05, **p < 0.01, indicates significance. . M+T: Combined MWCNT and TGF-β treatment.

4. Discussion

Multi-walled carbon nanotubes (MWCNTs) were initially studied for their potential fibrogenic activity shortly after their invention in 1991. Due to the propensity of small-scale fibers such as asbestos to cause inflammatory lesions and subsequent fibrotic foci, there was a large concern regarding potential human exposure [17]. Multiple studies have confirmed the fibrogenic potential of certain types of MWCNTs, but only a few studies have examined the effects of MWCNTs on cellular senescence [15, 16]. Pulmonary fibrosis is a disease of aging and senescence with a median age of diagnosis of 66 years of age [18, 19]. This study sought to evaluate whether exposure to MWCNTs would lead to cellular senescence and pro-fibrotic phenotypes in human bronchial epithelial cells (BEAS2B). In this study, we demonstrate that MWCNT (5μg/mL) exposure leads to an increased cellular ROS production, a reduction of cellular proliferation, and elevated levels of senescence markers (p21 and SA β-gal). Moreover, co-exposures with a fibrotic stimulus, TGF-β (5ng/mL), produced a more severe senescence phenotype, suggesting that MWCNTs induced senescence may be dependent on the cellular microenvironment.

Senescent cells accumulate naturally during aging in response to a number of external and internal stimuli such as DNA damage, metabolic alterations, and elevated ROS production [20]. Previous studies have reported that MWCNTs induce oxidative stress and can exhaust the antioxidant cellular capacity [21, 22]. Our results are consistent with these findings and demonstrate that MWCNTs are capable of disrupting redox biology, resulting in increased production of ROS. In addition, exposure of sub-confluence cultures of BEAS2B cells to MWCNTs over 48 hours resulted in cell growth arrest and inhibition of cellular proliferation. Evidence indicates that elevated levels of ROS may

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be required for certain types of premature senescence. In a previous report, induction of the cyclin dependent kinase (CDK) inhibitor p21 produced a robust increase in ROS resulting in cellular senescence in EJ cells. Moreover, N-acetyl-L-cysteine, a potent antioxidant can prevent p21 induced ROS accumulation and subsequent senescence [23, 24]. Here, we show that exposure to MWCNTs leads to an increased oxidative stress, an important factor mediating cellular senescence.

To further evaluate whether MWCNTs were leading to the development of cellular senescence we correlated our findings with established senescence biomarkers. Our data indicate that MWCNT exposure recapitulates some of the features of classical senescence such as an increase in SA β-gal activity and an increase in the protein abundance of p21 and slight increase in p16, but there was no change in γH2A.X, a sensitive indicator of DNA double strand (ds) breaks. In response to DNA ds breaks, Ataxia Telangiectasia Mutated (ATM) and other kinases recruit and phosphorylate H2A.X to ds breaks. This subsequently recruits other DNA repair mechanisms and triggers downstream p53/p21 CDKs to halt cell cycle progression. [25]. Accumulation of γH2A.X has been correlated with telomere shortening and induction of senescence. However, γH2A.X is observed in response to all ds breaks and doesn't necessarily converge to irreversible cell cycle arrest. Interestingly, many MWCNTs display little to no mutagenicity, possibly due to an induction of compensatory DNA repair mechanisms [15]. However, TGF-β can suppress DNA double strand repair mechanisms which is hypothesized to aid in genetic diversity in immune cells [26]. As a consequence, TGF-β may facilitate the mutagenic potential of MWCNTs. Indeed, co-exposure with MWCNTs resulted in an exacerbated senescence response with a significant increase in p16, p21 and γH2A.X protein levels compared to TGF-β alone. The effects of TGF-β and MWCNT on p21 and p16 appear to be additive, but the combined effects on γH2A.X suggest that synergistic effect may occur. TGF-β is known to induce cellular senescence and is sufficient to induce pulmonary fibrosis *in vivo* [27, 28]. Whether the observed combinatory effect of MWCNTs and TGF-β is a consequence of a unique interaction between them or results from breakdown of normal repair machinery due to multiple hits requires further investigation. MWCNT can activate SMAD proteins downstream of TGF-β [29] and may work in concert with added TGF-β to overstimulate the induction of senescence, but more research is needed to clarify these results.

To further investigate the potential combinatorial effects of TGF-β and MWCNTs, the EMT was evaluated. During EMT, epithelial cells lose cell polarity and contact adhesion molecules such as E-cadherin. There is not a clear consensus on the exact role of EMT in pulmonary fibrosis, but evidence suggests that EMT supplies the proliferation of myofibroblasts while other studies suggest that epithelial cells differentiate into mesenchymal support cells that stimulate the production of ECM proteins by activated myofibroblasts [30]. Previous reports indicate that MWCNTs can lead to a significant loss of E-cadherin [13], consistent with our findings. However, MWCNTs failed to increase production of ECM proteins fibronectin or plasminogen activator inhibitor 1 (PAI-1). Conversely, co-exposure of MWCNTs with TGF-β led to a marked increase in both fibronectin and PAI-1 compared to TGF-β alone. Interestingly, PAI-1 protein levels appear to be sensitive to the combination treatment. PAI-1 is a serine protease downstream of p53 and has been identified as a key mediator of cellular senescence. Fibroblast deficient in PAI-1 are resistant to cellular senescence [31] and knockdown of PAI-1 blunts TGF-β induced senescence [32]. Production of PAI-1 and Fn indicate that cells may be adopting a SASP. Collectively, these data suggest that MWCNT induced senescence may in part be dependent on the cytokine environment of the lung and that pro-fibrotic stimuli may interact with MWCNTs to propagate adverse cellular effects.

In conclusion, our study highlights the potential of MWCNTs to induce cellular senescence and provides new insight into its possible interactions with other senescent stimuli. While MWCNTs were only able to recapitulate some of the features of senescence, co-exposure with TGF-β resulted in a significant increase in cellular senescence and ECM production. Based on our data, the dysregulation of TGF-β signaling may promote MWCNT induced cellular senescence, which may be the underlying event in MWCNT-induced pulmonary fibrosis.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1.

Author Contributions: Conceptualization, J.L. and I.R.; methodology, J.L and Q.W.; Conducted experiments and data analysis, J.L; writing—original draft preparation, J.L.; writing—review and editing, J.L, Q.W, T.M. and I.R.; All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: We declare that we have provided all the data, but the primary data will be available upon request. Full unedited and uncropped blots are provided in the supplementary section.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Appendix A

Figure S1

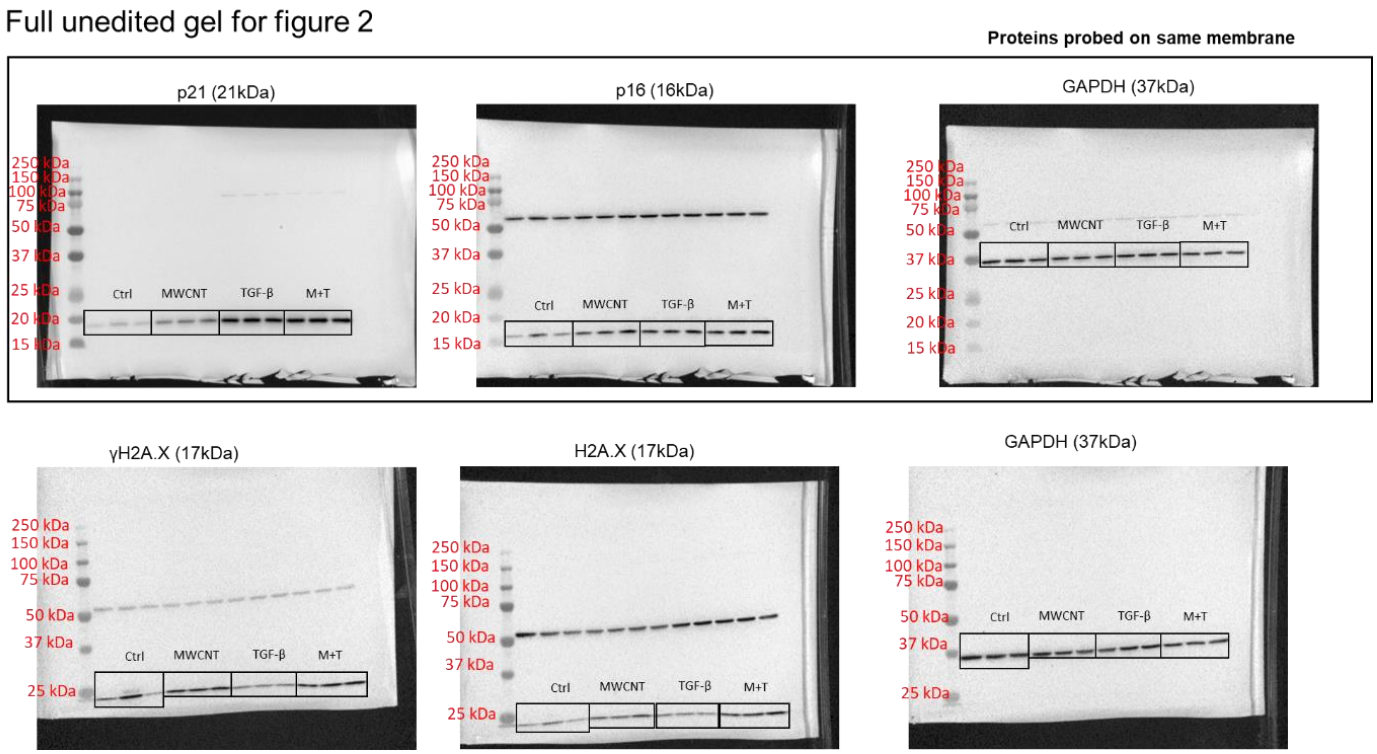

Figure S2

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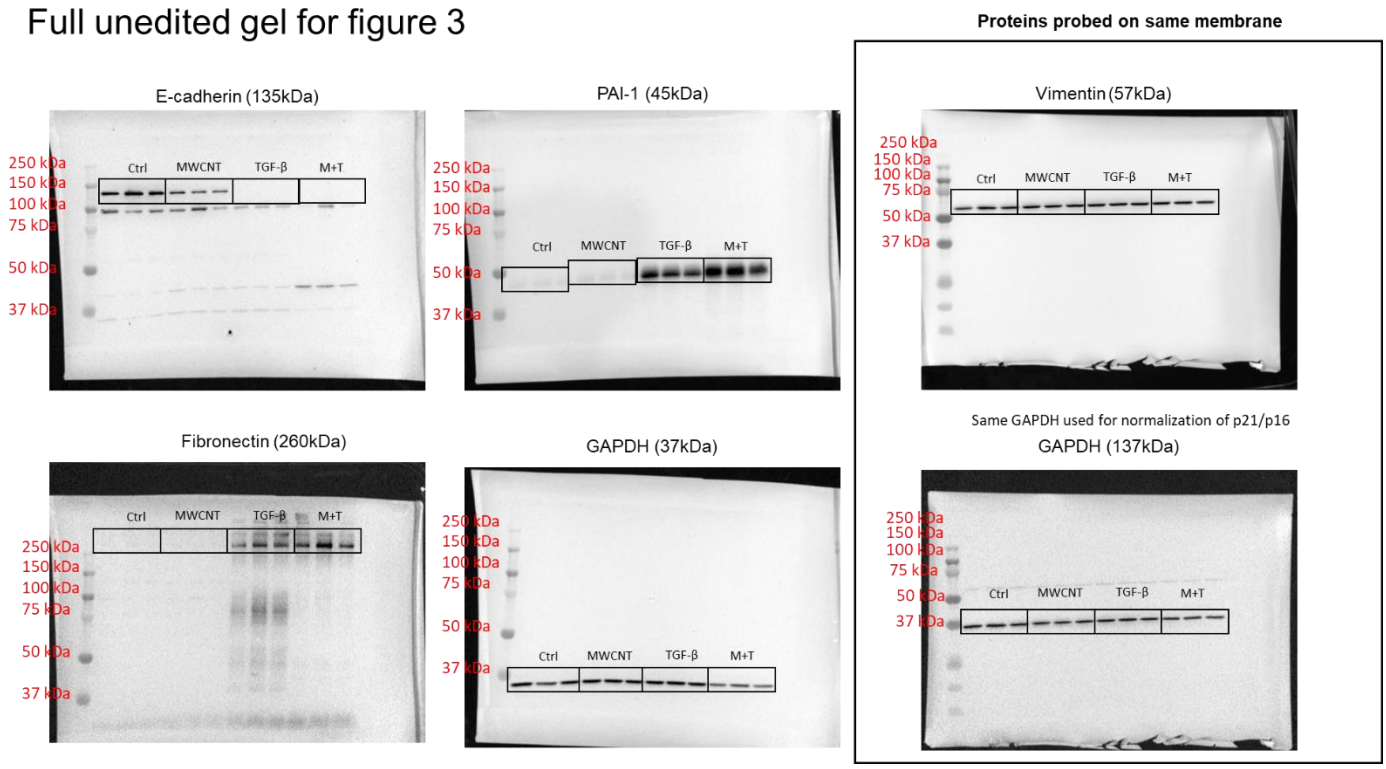

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