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Thrombospondin-1 and microRNA-1 Expression in Response to Multi-Walled Carbon Nanotubes in Alveolar Epithelial Cells

M Pacurari^{1,2,*}, R Kafoury^{1,2}, T Turner¹, S Taylor¹, and PB Tchounwou^{1,2}

¹Department of Biology, College of Science, Engineering, and Technology, Jackson State University, Jackson, MS, 39217, USA

²NIH/NIMHD RCMI Center for Environmental Health, College of Science, Engineering, and Technology, Jackson State University, Jackson, MS, 39217, USA

Abstract

Thrombospondin-1 (TSP-1) is a glycoprotein that plays a role in extracellular matrix (ECM) remodeling. Previously, we have shown that multi-walled carbon nanotubes (MWCNT) regulate ECM components TGF β and its target Col3A1 in alveolar epithelial cells. In the present study, we investigated the effect of MWCNT on TSP-1 and microRNA-1 (miR-1) in the regulation of TGF β in ECM remodeling using alveolar epithelial A549 cells. A549 cells were treated with MWCNT (20 or 50 μ g/ml) for 6 or 24 h and the expression of TSP-1 and miR-1, and the exogenous miR-1 effect on cell morphology were analyzed. MWCNT induced in a time- and dose-dependent manner the expression of TSP-1. miR-1 was suppressed by MWCNT after 6 or 24 h of treatment regardless of the dose. TSP-1 and miR-1 negatively correlated with each other, $r = -0.58$. Exogenous administration of miR-1 induced alveolar epithelial cell morphology changes including cell clustering, whereas inhibition of miR-1 induced less cell to cell contact, cell rounding, and cellular projections. IntAct molecular network interactions analysis revealed that TSP-1 interacts with 21 molecular factors including ECM genes, and molecules. These results indicate a relationship between that TSP-1, MWCNT and TGF β , and suggest TSP-1 may play a role in MWCNT-induced TGF β and ECM remodeling. Moreover, these data also suggest an inverse relationship between TSP-1 and miR-1 and a potential role of miR-1 in MWCNT-induced fibrotic signaling.

Introduction

Extracellular matrix (ECM) plays an important role in lung functioning (White, 2015). Multi-walled carbon nanotubes (MWCNT) have been shown to induce lung fibrosis in animal models (Ryman-Rasmussen et al., 2009; Mercer et al., 2013; also reviewed in Pacurari et al., 2016a). The exact molecular mechanisms by which MWCNT induce fibrosis are not fully understood, however molecular factors involved in MWCNT fibrotic signaling pathway are emerging. In lung fibroblasts, MWCNT induce the expression of TGF β receptor 1 (TGF β R1), TGF β 1, SMAD2/3 and collagen synthesis (Mishra et al., 2015). In alveolar epithelial cells, MWCNT induce the expression of ECM components TGF β and

* Correspondence to: Maricica Pacurari, PhD; maricica.pacurari@jsums.edu.

Col3A1, and remodeling zinc-metalloproteinases MMP-9 and MMP-12 (Pacurari et al., 2016). In support of the *in vitro* studies, the *in vivo* studies also showed the production of TGF β and MMP-9 in bronchoalveolar lavage cells (BAL) or whole lung lysate of mice treated with MWCNT (Mercer et al., 2013; Kasai et al., 2015; Aragon et al., 2016). In addition to activating TGF β -mediated fibrosis, MWCNT have been shown to also induce epithelial-mesenchymal transition (EMT) through TGF β -induced Akt/GSK/Snail-1 signaling (Polimeni et al., 2016).

Thrombospondin-1 (TSP-1) is an adhesive glycoprotein of ECM (Lawler et al., 1978). In ECM, TSP-1 interacts with numerous of its components including cell receptors, growth factors, and cytokines (Adams et al., 1995; Lawler et al., 1998; Resolvi et al., 2014). TSP-1 plays a role in many cellular and biological processes including cell migration and invasion, cell proliferation, cell adhesion, cell differentiation, inflammation, fibrogenesis, angiogenesis, and carcinogenesis (Azuma et al., 2005; Rico et al., 2007; Gutierrez, 2008; Kazerounian et al., 2008; Gianni and Guglielmo, 2010; Lawler and Lawler, 2012). Moreover, TSP-1 is a well-known activator of latent TGF β (Schultz-Cherry et al., 1994). TSP-1 plays a major role in lung wound repair and fibrosis through the activation of TGF β (Ezzie et al., 2010; Gianni et al., 2010; Sweetwyne and Murphy-Ullrich, 2012). Administration of a synthetic TSP-1 peptide to mice prevented the development of bleomycin-induced lung fibrosis (Chen et al., 2009). However, there is some emerging evidence suggesting in addition to TSP-1 there are other factors that may play a role in TGF β activation (Ezzie et al., 2011). Ezzie and co-workers found out that genetic deletion of TSP-1 did not prevent the development of subpleural fibrosis in mice treated with bleomycin.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression post-transcriptionally by binding to the 3'-untranslated region (3'-UTR) of target mRNAs and induce the degradation or inhibition of that particular mRNA (Liu et al., 2005; Pacurari et al., 2013). The exact role of miRNAs in MWCNT toxicity is less studied or understood. In nematode *Caenorhabditis elegans* (*C. elegans*), MWCNT differentially regulated 55 miRNAs, of which 5 miRNAs had a protective role and 7 miRNAs played a role in MWCNT toxicity (Zhao et al., 2014). In bronchoalveolar lavage cells and lung granulomas, MWCNT increased miRNA-33 expression (Barna et al., 2016).

Our previous study showed that MWCNT activate alveolar epithelial A549 cells to express fibrosis markers TGF β and Col 3A1. MWCNT induced TGF β by 2.62-, and Co3A1 by 2.34-fold change vs control (Pacurari et al., 2016b). Although, several studies have shown that MWCNT induce molecular factors associated with lung toxicity, the exact molecular factors involved in MWCNT signaling still remain unknown. Moreover little is known about MWCNT effect on microRNAs (miRNAs). Based on the observations that MWCNT regulate miRNAs and TSP-1 role in TGF β activation, and induce fibrosis, in the present study we investigated the effect of MWCNT on TSP-1 and miR-1 in alveolar epithelial cells.

Materials and Methods

Cell Culture

Alveolar epithelial A549 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cell cultures were maintained according to manufacturer's recommendations. Briefly, the cells were grown in Frank's Modified Medium (F-12K, ATTC) supplemented with 10 % fetal bovine serum (FBS), and Streptomycin/Neomycin (10,000/100 units, ATCC) in an incubator at 37 °C, 5% CO₂.

MWCNT preparation

For cell culture studies, MWCNT stock (2 mg/ml) were prepared in Ca²⁺/Mg²⁺-free phosphate-buffered saline (PBS), pH 7.4, according to the methods previously described (Pacurari et al., 2008; 2016). Briefly, MWCNT were prepared in PBS followed by indirect sonication at 4°C for 5 min (Hielscher ultrasonic processor, UIS259L) at amplitude 100% and cycle 1. After indirect sonication, the suspension was directly sonicated at 4°C for 1 min at 5W output and 10% duty cycle (Branson Sonifier 450). The stock solution (2 mg/ml) of MWCNT was kept at 4°C and used within 2–3 weeks. Prior to cell culture experiments, the MWCNT stock solution was directly sonicated for 1 min at the setting indicated above. Samples that were used for cellular studies were freshly prepared from the stock on the day of cell treatments to desired concentrations in RPMI containing 0.1% FBS and resonicated by a direct ultrasonication for 1 min before use.

Characterization of MWCNT

MWCNT were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO) and characterized as previously described in Pacurari et al. (2016b).

MicroRNA target prediction

miR-1 potential target gene identification was based on the method previously described by Pacurari et al. (2013) and Agrawal et al. (2015). To identify miR-1 potential target genes, we used TargetScan and microRNA.org applications (Betel et al., 2008; Pacurari et al., 2013; Agarwal et al., 2015). TargetScan is based on the presence of conserved 8-mer, 7-mer, or 6-mer sites that match the seed region of each miRNA (Agrawal et al., 2015). The microRNA.org uses miRanda algorithm to predict miRNA targets based on sequence and contextual features of the predicted miRNA-mRNA duplex (Betel et al., 2008). The consensus sites for microRNA-mRNA binding are presented according to *Homo sapiens* microRNA.org database using search for 'Target mRNA' module with parameters for mirSVR score 0 and PhastCons score 0.

RNA isolation

Total RNA was extracted using Trizol[®] (Invitrogen, CA) according to the manufacturer's protocol. To ensure a good RNA quality, the quality and integrity of the total RNA was evaluated using 28S/18S ratio and a visual image of the 28S and 18S bands were evaluated on the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Concentration of the total

RNA was assessed using the NanoDrop-1000 Spectrophotometer (NanoDrop Technologies, Germany).

Quantitative real-time (q)PCR

For miRNA analysis, complementary DNA (cDNA) was generated using total RNA isolated as described above and according to the TaqMan® MicroRNA Reverse Transcription protocol (Applied Biosystems Inc., Carlsbad, CA). RT-PCR for microR-1 was performed using TaqMan MicroRNA assays (Applied Biosystems Inc., Carlsbad, CA). Human U47 small nuclear RNA was used as an endogenous control. The expression levels of miRNAs were quantified using ABI 7500 quantitative real-time instrument and SDS software (Applied Biosystems Inc., Carlsbad, CA). The abundance of miRNA is expressed as Ct (threshold fluorescence), which gives the number of cycles required to reach threshold fluorescence. qPCR for miR-1 potential target gene was determined using total RNA. cDNA was generated using a High-Capacity cDNA Reverse Transcription kit and TaqMan gene expression assays (Applied Biosystems Inc., Carlsbad, CA). The levels of TSP-1 and 18S mRNA was measured using SYBR-Green Master mix and gene specific primers according to manufacturer's protocol (Applied Biosystems Inc., Carlsbad, CA). All qRT-PCR reactions were performed on 7500 Fast Real-Time PCR instrument (Applied Biosystems Inc., Carlsbad, CA). In the qRT-PCR analysis of genes, the dissociation curve showed the absence of a secondary peak, indicating no presence of primer dimer. The expression level of each gene was determined by following formulas: fold change = 2^{-Ct} , where Ct (cycle threshold) = $Ct_{\text{target gene}} - Ct_{\text{endogenous control gene}}$, and $Ct = Ct_{\text{treated sample}} - Ct_{\text{control sample}}$. 18S was used as an endogenous control gene (Pacurari et al., 2011; 2013).

TSP-1 immunofluorescence

Immunofluorescence was used to analyze TSP-1 expression (He et al., 2011, Pacurari et al., 2012). Briefly, the cells were grown on cover slips and treated with MWCNT for 6 or 24 h. After the treatments, the cells were fixed in 4% paraformaldehyde and permeabilized in 0.1% TritonX-100/PBS. Cells were incubated with 5% bovine serum albumin (BSA)/PBS/0.1% TritonX-100 for 2 h at room temperature and then incubated with anti-TSP-1 antibody (ThermoFisher Scientific, Carlsbad, CA) in 5% BSA/PBS/0.1% TritonX-100 at 1:500 dilution overnight at 4°C. After primary incubation, the cells were washed three times with PBS and then incubated with an Alexa-Fluor®-488-conjugated secondary antibody (Molecular Probes, Life Technologies, MA)/5% BSA/PBS/0.1% TritonX-100 at a dilution of 1:1000 for 1 hour in the dark. After secondary antibody incubation, the cells were washed with PBS three times for 5 min each time, and mounted with a mounting antifade solution containing DAPI (Molecular Probes, Eugene, OR). Images were taken with an Olympus epifluorescence microscope (Center Valley, PA).

Western blot

After treatments, the cells were lysed in 1X SDS lysis buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol). Total protein was quantified by the BCA method. β-mercaptoethanol was added to lysates to a final concentration 100 mM. Equal amounts of total protein were separated by 4–12% SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked with 5% non-fat milk in 1X PBS containing 0.05% Tween-20 for 1 h at room

temperature. Membranes were then incubated with primary antibody at 4°C overnight. After the incubation the membranes were washed three times in 1X PBS with 0.05% Tween-20 for 10 min and then incubated for 1 h at room temperature with horseradish peroxidase (HRP) conjugated goat anti-mouse IgG in 5% non-fat milk/1X PBS/ 0.05% Tween-20. Membranes were then washed five times for 10 min in 1X PBS with 0.05% Tween-20 and the proteins were visualized using an ECL Chemiluminescence Kit (Millipore, MA). Relative protein level was determined after normalization to beta-actin and relative to negative control (miR-scr) samples.

Wound scratch assay

Wound healing assay was performed according to the methods previously described (Pacurari et al. 2012; Ju et al., 2014; Pacurari et al., 2016b). A549 cells were grown on cover slips to a confluent monolayer and then scratched to form a 100- μ m “wound” using 100 μ L sterile pipette tips. The cells were treated with MWCNT (20 or 50 μ g/ml) in serum-free media for 24 h, washed one time with PBS and then fixed in 4% paraformaldehyde. Samples were imaged using an Olympus IX70 microscope (Olympus Optical Co., Ltd, Japan) equipped with a Retiga 2000R FAST camera (Qimaging, Canada). Images were acquired using SimplePCI software (Compix Inc., Sewickley, PA).

MiR-1 transfection

The cells were transiently transfected with miR-1 mimic (50 nM) or miR-1 inhibitor (50 nM), and their corresponding negative controls (Ambion, Naugatuck, CT) using Lipofectamine™ 2000 (Invitrogen, CA) in Opti-MEM (Invitrogen) according to the manufacturer’s recommendations. The negative control (NC) scrambled oligonucleotide does not encode for any known miRNA.

Functional pathway analysis

Molecular network interactions were performed using European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) and as previously described in Pacurari et al., 2013. EMBL-EBI offers an array of up-to date molecular databases. In the present study, we used EMBL-EBI IntAct molecular interactions database which freely provides open sources for database systems and analysis tools for molecular interactions. Network interactions are derived from literature curation or direct user submitted information (www.ebi.ac.uk/intact/). Numerous studies have used IntAct molecular network interactions (Orchard et al., 2014; Zhang et al., 2015). The molecular network interactions include both physical and functional interactions, and novel molecular targets that are reported in the IntAct database.

Statistical analysis

Data are presented as mean $\pm$ SEM from three experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) and Student’s paired *t*-test with a significance level of *p* = 0.05 versus non-treated control samples.

Results

Identification of Mir-1 target gene

To identify a potential miR-1 target gene, we used TargetScan and microRNA.org engines to determine whether or not lung fibrosis and remodeling associated genes are predicted targets for miR-1 (Fig. 1A). TargetScan identified 2456 potential target genes and microRNA.org identified 7204 potential target genes for miR-1 including fibrosis and ECM remodeling genes such as Col3A1, Col13A1, Col19A1, TGIF1, Adams 9, and TSP-1. Using TSP-1 as a searching query in the microRNA.org database, it was found that TSP-1 contains three miR-1 binding sites (Fig. 1B) with a combined mirSVR score for all target miR-1 sites of -1.1233 . mirSVR score represents the likelihood of an mRNA downregulation by a miRNA based on regression analysis on the targets site for a miRNA in an mRNA with a 6-mer or better seed site, or a mirSVR score -0.1 (Betel et al., 2010).

MWCNT effect on TSP-1

TSP-1 mRNA was not significantly increased by 20 $\mu\text{g/ml}$ of MWCNT after 6 h of treatment, a 1.13-fold change vs control, whereas 50 $\mu\text{g/ml}$ significantly increased TSP-1 mRNA by 2.25-fold versus control (Fig. 2A). After 24 h, MWCNT at both doses, 20 and 50 $\mu\text{g/ml}$, significantly increased TSP-1 mRNA by 2.59- and 2.28-fold versus control, respectively (Fig. 2A). Immunohistochemistry analysis of TSP-1 following MWCNT treatment revealed a strong immunodetection of TSP-1 (green) in MWCNT treated samples compared to control samples (Fig. 2B). Immunohistochemistry analysis showed a strong TSP-1 detection at cell periphery particularly after 6 h of treatment with 50 $\mu\text{g/ml}$ MWCNT (Fig. 2B, arrow). Western blot analysis showed an increase of TSP-1 protein level following 6 h with 50 $\mu\text{g/ml}$ MWCNT and after 24 h of treatment with both doses compared to control (Figs. 2C–D).

MWCNT effect on MiR-1

Analysis of miR-1 in alveolar epithelial A549 cells following MWCNT treatment revealed a significant inhibition of miR-1 expression relative to control (Fig. 3). MWCNT dose-dependently inhibited miR-1 expression. MWCNT at 20 $\mu\text{g/ml}$ after 6 h of treatment inhibited miR-1 expression by 75%, whereas after 24 h of treatment with 20 $\mu\text{g/ml}$ of MWCNT, miR-1 level was inhibited only by 60% relative to control. MWCNT at 50 $\mu\text{g/ml}$ after 6 h of treatment inhibited miR-1 by 80% while after 24 h of treatment, miR-1 was almost undetectable (Fig. 3). Since TSP-1 was found to be a predicted target of miR-1, we performed a correlation analysis between the expression level of TSP-1 and miR-1 (Fig. 4). Pearson correlation analysis found a strong inverse relationship between miR-1 and TSP-1 mRNA with $r = -0.58$.

MWCNT effect on cell migration

We further analyzed MWCNT dose-dependent effect on cell migration toward wound healing after 24 h of treatment. MWCNT at 20 or 50 $\mu\text{g/ml}$ stimulated a weak cell migration toward wound healing (Fig. 5A). MWCNT at 20 and 50 $\mu\text{g/ml}$ stimulated cell migration by 1.67- and 1.49-fold vs control (Fig. 5B). Previously, we showed a higher cell migration in

response to 20 µg/ml MWCNT after 48 h (Pacurari et al., 2016b). These results suggest that MWCNT-induced cell migration is a complex process that is temporally and spatially regulated process.

Ectopic MiR-1 effect on cell morphology

The effect of transient expression or inhibition of miR-1 on cell morphology was analyzed by transfecting the cells with miR-1 mimic or miR-1 inhibitor. Ectopic miR-1 mimic induced cell morphology changes such as cell clustering with cell making contact with each other. By contrast, ectopic miR-1 inhibitor induced less cell to cell contact where cells separated from each other, and displayed an elongated morphology with cell periphery projections (Fig. 6; cell rounding = arrow; spindle shaped = dotted arrow).

Molecular network analysis

Molecular network interactions associated with TSP-1 and its interacting proteins were retrieved using IntAct (Fig. 7). The molecular network map indicates that TSP-1 interacted with other 16 genes, 4 polysaccharides, and 2 molecular factors, ssRNA_AG and Q9WMX2-pro_0000037551, for which the function are still unknown. Among 16 genes interacting with TSP-1, we identified TGFβ, collagen types (Col 1A1 and its paralog Col3A1, Col 11A1, Col VI), FN1, BGN, TGM2, and TUBB5 (Table 1). Majority of the identified 16 genes are components of the ECM and involved in signaling pathways associated ECM organization, collagen biosynthesis, cell adhesion, and cell-to-cell communication, and TGFβ signaling pathway. The identified polysaccharides include heparan sulfate, chondroitin sulfate, and dermatan sulfate which are known components of proteoglycans found on cell surface and in the ECM (Sasisekharan et al., 2000). In addition, TSP-1 also interacted with heparin which is a known anti-clotting factor, however the exact physiological role of heparin is not fully known, and some studies suggest that heparin might play a role in catalytic activation of FN within ECM (Sasisekharan et al., 2000; Mitsi et al., 2008).

4. Discussion

In the present study, we analyzed MWCNT effect on TSP-1, miR-1, and ectopic miR-1 mimic and inhibitor effect on alveolar epithelial A549 cells. TGFβ signaling is critical in tissue homeostasis and morphology not only during normal embryonic development and adulthood, but also during tissue injury and wound healing. Numerous studies previously have shown that MWCNT induce lung fibrosis in experimental animal models (Mercer et al., 2013; Mishra et al., 2015; Wang et al., 2015). The fibrotic potential of MWCNT is mediated by the activation of TGFβ signaling pathway (Mishra et al., 2015; Wang et al., 2015; Pacurari et al., 2016b). Mishra et al. (2015) showed that MWCNT activate TGFβ signaling pathway by up-regulating the expression of its receptor, TGFβ R1, and downstream activation of Smad2/3 proteins. TGFβ regulates pro-fibrotic genes in lung residing fibroblast and alveolar epithelial cells, and thus induces remodeling of ECM (Sheppard D., 2006; Pacurari et al., 2016b; Polimeni et al., 2016). Wang et al. (2015) showed that MWCNT induce fibroblast to myoblast and EMT through TGFβ/Smad signaling. Recent studies indicate that EMT plays a role in lung fibrosis. Previously, we

showed that exposure of alveolar epithelial A549 cells to MWNCT resulted in an increased expression of TGF β and its downstream target gene Col3A1. We also showed that MWCNT induced the expression of ECM remodeling genes MMP-9 and MMP-12 (Pacurari et al., 2016b). In the present study, we show that MWCNT increase the expression of TSP-1 and suppresses the expression of miR-1 (Fig. 2 and Fig. 3). TSP-1 plays an important role in normal lung homeostasis (Lawler et al., 1998). Also, TSP-1 is a known key activator of TGF β (Ribeiro et al., 1999). As an adhesive glycoprotein, TSP-1 localizes to ECM and interacts with matricellular components including cell receptors, growth factors, and cytokines, and thus regulating many biological processes including cell proliferation, cell migration, and invasion (Adams et al., 1995; Lawler et al., 1998; Lawler and Lawler, 2012; Resolvi et al., 2014). In lung pathologies such as wound repair and fibrosis, TSP-1 has been shown to play a role via TGF β activation (Daniel et al., 2004; Gianni et al., 2010; Sweetwyne and Murphy-Ullrich, 2012). Moreover, MWCNT have been shown to stimulate TGF β production by lung alveolar and fibroblast cells (He et al., 2011; Chen et al., 2014; Mishra et al., 2015; Pacurari et al., 2016b). The data in the present study suggests also a role of TSP-1 in TGF β signaling in response to MWCNT in alveolar cells. Although, fibroblasts are central to fibrosis, other lung cells including alveolar epithelial cells are emerging contributors to fibrosis development (Strieter and Mehrad, 2009; Dong et al., 2016). Treatment of alveolar epithelial cells treated with TGF β induces the expression of EMT markers, vimentin and α -smooth muscle actin, or collagen type I (α -SMA) (Hisatomi et al., 2012; Dong et al., 2016). Several other studies indicate EMT role in lung fibrosis (Willis et al., 2005; Hisatomi et al., 2012). Previously, we showed a MWCNT dose-dependent effect on TGF β expression. By increasing MWCNT dose from 20 to 50 μ g/ml, TGF β increased by 2.12 times (Pacurari et al., 2016b). Corroborating the data of this study and previously published studies, we suggest a role of TSP-1 and TGF β in MWCNT fibrotic potential. The data in the present study suggest that TSP-1 plays a role in MWCNT activation of alveolar epithelial cells.

Using bioinformatics and gene databases, we previously identified new targets in metastatic lung cancer cells: miRNAs-200a, -200b, and 200c (Pacurari et al., 2013). In the present study, using TargetScan and mirorna.org, we have identified TSP-1 to be a target for miR-1 regulation. We have shown that MWCNT significantly inhibited miR-1 expression. The inhibitory effect was dose-dependent since MWCNT after 6 h of treatment MWCNT at 20 μ g/ml decreased miR-1 by 75% and at 50 μ g/ml miR-1 was decreased by 80% compared to the control (Fig. 3). In the present study, we have also found TSP-1 expression to be inversely correlated with miR-1, showing a negative correlation coefficient, $r = -0.58$. The lack of immediate response in TSP-1 mRNA by MWCNT 20 μ g/ml after 6 h of treatment although we did find a downregulation of miR-1 suggests that gene expression regulation is complex, and perhaps depending on the type of insult, and perhaps temporally regulated. Similar to our findings, although in a different context, Bryant et al. (2012) showed that when A549 cells were co-treated with miR-200c and TGF β 1, an opposite effect on EMT proteins and miR-200c itself was observed as opposed to when the cells were treated only with TGF β 1.

The role of miRNAs in MWCNT signaling is relatively unknown. Zhao et al. (2014) showed that 55 miRNAs play a role in MWCNT toxicity in *C. elegans*. Among the 55 miRNAs

regulated by MWCNT, miR-1 was one of the 34 miRNAs that were downregulated. To test the role of targeted microRNA by MWCNT, Zhao et al. used specific MWCNT-targeted microRNA mutants. For example, miR-45, miR-51, and miR-35–41 mutants showed increased reactive oxygen species (ROS) formation. Moreover, miR-259 mutant nematodes exhibited MWCNT toxicity, whereas miR-51 mutants exhibited resistance toward MWCNT toxicity. The study by Zhao et al., (2016) suggest a role of miRNAs in MWCNT toxicity and that MWCNT-induced ROS regulates miRNAs. In the present study, we show a profound down-regulation of miR-1 by MWCNT. Presumably, signaling molecules such as ROS that are produced by cells in response to MWCNT immediately interact with cellular components including miRNAs and the effect of MWCNT can be detected early, as shown in the present study where MWCNT at 20 or 50 µg/ml profoundly altered miR-1 level. Our previous and other studies showed ROS production by MWCNT, thus a role of MWCNT via ROS production in TGFβ signaling via miR-1 and TSP-1 may play a role, yet the exact mechanism of miR-1 downregulation by MWCNT remains to be investigated in further studies (Xe et al., 2011; Pacurari et al., 2012; Patel et al., 2012). Furthermore, Patel et al. (2012) showed that exposure of A549 cells to 5, 10, or 20 µg/ml of MWCNT, only 20 µg/ml of MWCNT induced ROS. However, after 48 h of exposure, all concentrations of MWCNT increased ROS, and after 72 h only 20 µg/ml increased ROS which was slightly higher than 48 h but lower than 24 h. These results indicate an intricate MWCNT-induced signaling and also support our miR-1 expression in response to MWCNT.

Moreover, there is growing evidence indicating that miR-1 is downregulated in several cancer types including lung (Truini et al., 2016; Liu et al., 2015; Yang et al., 2016). MiR-1 re-expression targets tumorigenesis by modulating c-Myc, cMet, FoxP, Pim-1, PI3K/Akt, and Wnt/β-catenin signaling pathway and thus inhibiting cell proliferation and migration (Nasser et al., 2008; Kojima et al., 2012; Yu et al., 2014; Liu et al., 2015). In the present study, we observed morphology changes of cells treated with miR-1 inhibitor, particularly, loss of cell to cell contact and the presence of membrane protrusions, characteristics of migratory cells which are in agreement with other studies which showed that re-expressing miR-1 limits cell migration and invasiveness (Nasser et al., 2008; Kojima et al., 2012; Liu et al., 2015). In the present study, MWCNT induced a weak cell migration toward wound healing after 24 h, results that are in agreement with other studies (Raffa et al., 2009; Ju et al., 2014). Numerous studies have shown that MWCNT support both cell migration and invasion, and that cell migration is over-stimulated after a longer period of exposure to MWCNT (Lohcharoenkal et al., 2013; Ju et al., 2014; Pacurari et al., 2016b). Cell migration is a complex process that is spatially and temporally regulated by many molecular factors involved cell structure, cell adhesion and interactions with ECM components (Lauffenburger et al., 1996; Hurd et al., 2012), and thus the results of the present study suggest a time-dependent effect of cell migration in response to MWCNT.

MWCNT have been shown to induce fibroblast-myoblast and EMT via the activation of TGFβ/Smad in A549 cells, and alveolar epithelial cells are emerging as key players in pulmonary fibrosis (Wang et al., 2015). Previously it has been shown that MWCNT stimulate cell migration, and in the present study we have shown a down-regulation of miR-1 by MWCNT, thus suggesting a potential role of miR-1 downregulation by MWCNT and stimulation of cell migration. Although the exact role of miR-1 and TSP-1 in cell

migration is not known yet, studies have shown that the formation of membrane protrusions such as filopodia and lamellipodia contributes to TGF β -mediated cell migration (Tominaga et al., 2012; Polimeli et al., 2016). MWCNT have been shown to induce the formation of filopodia and lamellipodia (Pacurari et al., 2011). Moreover, studies have shown that TSP-1 enhances cell invasion by up-regulating the activity of MMP-9 (Qian et al., 1997; Albo et al., 2002). Studies have shown increased expression of MMP-9 and MMP-12 in response to MWCNT, thus suggesting a relationship between TSP-1 and ECM remodeling in response to MWCNT stimulation (Aragon et al., 2016; Pacurari et al., 2016b). The present study suggests a role of miR-1 in MWCNT-induced TGF β signaling via TSP-1. Increased expression of TSP-1 has been implicated in EMT along with increased cell migration (Jayachandran et al., 2014). Considering that TSP-1 is a major activator of TGF β , the data presented in this study indicate a role of TSP-1 in MWCNT-induced TGF β signaling and lung fibrosis, and suggest a role of miR-1 in MWCNT-induced TSP-1 which is further investigated in the ongoing laboratory studies.

5. Conclusion

The results of the present study indicate a role of TSP-1 in MWCNT-induced ECM remodeling. They also show that MWCNT alter the expression of miR-1 in alveolar epithelial cells. Studies have shown that TSP-1 activates TGF β and hence plays a role in fibrosis. The present results also indicate a relationship between miR-1 and TGF β signaling, thus suggesting a role of miR-1 and TSP-1 in response to MWCNT modulation of lung ECM remodeling and fibrosis. These results taken together with findings from previously published studies suggest a possible mechanism for MWCNT-induced lung remodeling and fibrosis through miR-1, TSP-1, and TGF β . We have previously reported that MWCNT induce the formation of ROS, activate NF- κ B, and increase the expression of MMP-9 and MMP-12 (Pacurari et al., 2011; Pacurari et al., 2016b). Corroborating our previously published studies with the present study, MWCNT-induced lung ECM remodeling and fibrosis involves many steps including increased expression of TSP-1, and its localization to plasma membrane. In parallel, MWCNT also induce the expression of TGF β , which becomes activated by TSP-1 and subsequent activation of TGF β . The activated TGF β signals downstream and induces the synthesis of Col3A1, and genes associated with ECM remodeling and lung fibrosis.

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Fig. 1.

A) Bioinformatics prediction model for miR-1 and its target gene associated with lung fibrosis/remodeling and assay for miR-1 and target gene. **B)** 3' - UTR sequence of miR-1 putative binding sites to target gene TSP-1 is given in the 5' - to 3' - orientation.

Fig. 2. TSP-1 regulation by MWCNT. **A**) TSP-1 mRNA level in the cells treated with MWCNT (20 or 50 $\mu\text{g/ml}$) for 6 or 24 h. The data is presented as mRNA fold change relative to 18S. The data represents the mean $\pm$ SEM, $n=3$. **B**) TSP-1 immunohistochemistry detected using TSP-1 immunofluorescence (green) and DAPI (nucleus). The arrow points to

immunofluorescence of TSP-1. **C)** Western blot analysis of TSP-1 and beta-actin in A549 cells treated with MWCNT as indicated in the figure. One representative blot is shown. **D)** TSP-1 intensity analysis normalized to beta actin and relative to control untreated samples. The data represents the mean $\pm$ SEM, n=3. *Statistically significant versus control, ANOVA, $p < 0.05$.

Fig. 3.

The effect of MWCTN on miR-1 level. The cells were treated with MWCNT, 20 or 50 µg/ml, for 6 and 24 h. MicroRNA was detected using real-time qPCR according to TaqMan assay as described in Materials and Methods. The data shown represents the mean $\pm$ SEM, n=3. *Statistically significant versus control, ANOVA, p < 0.05.

Fig. 4. Correlation analysis between miR-1 and TSP-1 mRNA. The data were analyzed using Pearson correlation analysis between TSP-1 mRNA fold change and miR-1 fold change.

Fig. 5. Cell migration in wound healing after exposure to MWNT (20 or 50 $\mu\text{g/ml}$) for 24 h. **A).** The cell monolayer was wounded using wound scratch assay and cell migration toward wound healing was observed after 24 h. One representative micrograph is shown. **B).** Counted migrating cells toward wound healing. Data is presented as mean $\pm$ SEM, $n=3$. *Statistically significant versus control, ANOVA, $p < 0.05$.

Fig. 6. Comparison of cell morphology after microRNA-1 overexpression or inhibition. **A)** Control; **B)** miR-1 mimic; **C)** miR-1 inhibitor. The cells were seeded in growth media for 24 h followed by treatments with miR-1 mimic and miR-1 inhibitor using lipofectamine in basal media for 48 h. Representative micrographs of one experiment, n=3.

Fig. 7. Molecular network analysis of TSP-1 interactions with other molecular targets. The network was generated using IntAct. The map shows that TSP-1 interacts with 22 other genes and molecules of ECM including TGF β and Col.

Table 1

Information on molecular network interacting genes containing gene name, gene symbol, cellular function, signaling pathways, and NCBI references.

Gene name	Gene symbol	Cellular function	Signaling pathway	NCBI Reference
Collagen Type 1 and its paralog Collagen Type 3	Col 1 Col 3	Fibrillar forming collagen of soft tissues	ECM organization, Focal adhesion, Collagen biosynthesis, Platelet activation	NM_000088 NM_000090
Collagen Type VI	Col VI	Microfibrillar forming collagen	ECM organization, Focal Adhesion, ECM-receptor interaction	NM_001848
Collagen Type 11 Alpha 1	Col 11A1	Fibrillar forming collagen	ECM organization, Focal Adhesion, Collagen biosynthesis	NM_080629
Collagen Type 18 Alpha 1	Col 18A1	Multiplexin binding protein	ECM organization, Collagen biosynthesis	NM_030582
APP	Amyloid beta Precursor protein	Cell surface receptor, Cell adhesion	Homeostasis, Platelet signaling	NM_00120430
Biglycan	BGN	Collagen fiber assembly	Degradation of ECM, Metabolism	NM_001711
Fibronectin 1	FN1	Cell adhesion and migration in wound healing and metastasis	ECM organization, Regulation of actin cytoskeleton, Focal adhesion	NM_00130613
PCOLCE	Procollagen C-endopeptidase enhancer	Enzymatic cleavage of type I procollagen	Degradation of ECM, Collagen synthesis and modifying enzymes	NM_002593
Pleckstrin	PLEK	Protein homodimerization	Platelet activation, signaling, and aggregation	NM_002664
Thrombospondin 1	(THBS1, TSP-1)	Cell-to-cell interactions, Cell-to-matrix interactions, Focal adhesion	Platelet activations, signaling, and aggregation, TGF β Signaling pathway, ECM organization	NM_003246

Gene name	Gene symbol	Cellular function	Signaling pathway	NCBI Reference
Transforming growth factor beta	TGFβ	Cell proliferation, differentiation, migration, and many other functions in many cell types, Epithelial-to-Mesenchymal transition (EMT)	TGFβ Signaling pathway, Cell cycle, ECM organization, Homeostasis	NM_000660.5
Transglutaminase	TGM 2	Proteins crosslinking, Polyamines conjugation to proteins	PLC signaling CREB signaling	NM_004613
Tubulin Beta Class 5	TUBB5	Microtubules structure	Cytoskeleton remodeling, Cell cycle, Gap junctions	NM_00129321
SSRNA_AG				
IQCB1	IQ Calmodulin-Binding Motif Containing 1	Calmodulin binding, Ciliary function	Organelle biogenesis and maintenance	NM_00102357