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Toxicogenomics of Multi-Walled Carbon Nanotubes

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Introduction

The International Standards Organization (ISO) defines nanomaterial as "material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale" (ISO 2015). Nanoscale is defined as the size ranging approximately from 1 to 100 nm (ISO (International Standards Organization) 2015). Materials having at least one dimension in the nanoscale are considered by the US National Nanotechnology Initiative as nanomaterials (Yokel and MacPhail 2011). Nanomaterials or nanoparticles can be either naturally occurring or manufactured. Nanomaterials that are manufactured for specific purposes are commonly referred to as engineered nanomaterials (ENMs). ENMs are considered as advanced materials that are specifically engineered with unique and desirable properties intended for superior performance compared with conventional materials. ENMs can be generated by bottom-up processes, such as physical and chemical vapor deposition, liquid phase synthesis, and self-assembly, starting with chemical elements, such as carbon, metals, metal oxides, polymers, and biological molecules (Cao 2004). They can also be generated through top-down approaches by breaking down bulk materials to the nanoscale. ENMs exhibit many desirable properties that include, but are not limited to, their nanoscale size, light weight, and increased surface area, stiffness, tensile strength, and stability. Additionally, ENMs may exhibit superior electromagnetic, catalytic, thermal, and pharmacokinetic properties. The unique and desirable properties of ENMs have facilitated their use either directly or in products enabled by them. ENMs have found numerous commercial, industrial, and medical applications (De Volder et al. 2013; Eatemadi et al. 2014; Stoner et al. 2014). These include, but are not limited to, their use in cosmetics, personal care products, drug delivery, electronics, automobiles, and household and home improvement products. The widespread use of ENMs in products presents numerous opportunities for their exposure among workers who are engaged in the manufacture of such products and consumers who use them. Many of the same desirable properties of ENMs that enable their use in commerce, industries, and medicine have also raised serious concern from a human health and safety standpoint. For example, due to their light weight, ENMs can be easily aerosolized with potential for inhalation exposure among workers who are engaged in the manufacture and use of ENMs or ENM-containing products. Similarly, the size of ENMs in the nanoscale may enable their entry into deep anatomical areas in the lungs where interaction with cellular components may result in biological activity that may be quantitatively and/or qualitatively distinct from their large-sized counterparts of similar chemical composition (Castranova

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2011). ENMs may enter the circulatory system from the lungs and translocate to other organs to result in systemic toxicity (Simeonova and Erdely 2009). Protection from adverse health effects in workers and consumers who are likely to be exposed to ENMs is a priority for various government and nongovernment agencies and organizations. This requires a clear understanding of the toxicity potential of ENMs and the mechanisms underlying the toxicity that are integral components in the assessment and management of the potential health risks associated with human exposure to ENMs. Current knowledge with respect to the lung toxicity caused by multi-walled carbon nanotubes (MWCNT), perhaps the most studied ENM, is presented in this chapter with an emphasis on the application of transcriptomics in investigating ENM-induced lung toxicity.

MWCNTs

MWCNTs are carbon-based nanomaterials consisting of multiple concentric layers of graphene sheets composed of carbon rings rolled into cylindrical structures. Typically, the diameter of a MWCNT is in the nanoscale and the length can range from a few nanometers to several millimeters or more. MWCNTs, due to their many desirable features, such as light weight, increased surface area, tensile strength, and stiffness and enhanced durability, have found multiple applications in commerce, industry, and medicine (Eatemadi et al. 2014; Stark et al. 2015; Stoner et al. 2014). Unfortunately, the same desirable characteristics that have facilitated the use of MWCNTs in commercial, industrial, and medical applications have raised concerns with respect to their biological activity that can result in adverse health effects. Some MWCNTs, because of their resemblance to asbestos with respect to the thin and needle-like shape, rigidity, and biopersistence, have raised concerns about the potential for cancer and mesothelioma (Donaldson et al. 2010; Fukushima et al. 2018; Kasai et al. 2016). Significant variations in size, shape, length, thickness of the wall, aspect ratio, presence of metal contaminants and their levels, and surface chemistry exist among the various preparations of MWCNTs, all of which are known to significantly influence the potential of MWCNTs for differing degrees of toxicity, and these are known to exist among the various preparations of MWCNTs (Johnston et al. 2010; Fraser et al. 2020, 2021).

MWCNTs, primarily due to their lightweight, can be easily aerosolized, and the airborne particles may present a significant risk for inhalation exposure to result in pulmonary and systemic toxicity in humans. In an exposure assessment study (Erdely et al. 2013), the mass concentration of elemental carbon present in the personal breathing zones (PBZ) of workers employed in eight US-based facilities manufacturing MWCNTs was determined as an index of their occupational exposure to MWCNTs. The inhalable elemental carbon mass concentration in PBZ samples collected exhibited an arithmetic mean of 10.6 $\mu g/m^3$ (geometric mass 4.21 $\mu g/m^3$), suggesting the presence of airborne MWCNT particles and their potential exposure among workers employed in the manufacture of MWCNTs. In another study conducted by Han et al. (2008), the total mass of all particulates in full shift air samples collected from a research facility handling MWCNTs was determined. The total mass of all the particulates in the PBZ samples collected from the facility ranged from non-detectable concentrations to 331.7 $\mu g/m^3$, further supporting the presence of airborne MWCNT particles in workplaces with potential human exposure. In a cross-sectional study, Dahm et al. (2018) measured exposure to carbon nanotubes and fibers among 108 participants enrolled from 12 facilities across the United States. Mean exposure to elemental carbon (EC) at the respirable and inhalable size aerosol fractions collected from the facilities was 1 and 6.22 $\mu g/m^3$, respectively. Additionally, ENM particles were detected in 18% and 70% of the sputum and hand/dermal samples, respectively, collected from the study participants, confirming

exposures. Similar findings supporting the presence of airborne MWCNT particles in workplaces with potential human exposure have been reported in other studies (Dahm et al. 2012, 2013, 2015; Hedmer et al. 2014; Fatkhutdinova et al. 2016; Kuijpers et al. 2016; Shvedova et al. 2016; Lee et al. 2015; Vlaanderen et al. 2017). Currently, to protect workers from the potential adverse health effects associated with occupational exposure to the MWCNTs, NIOSH recommends that exposure to the ENM should be kept below an 8-hour time-weighted average of 1 $\mu g/m^3$ of respirable EC (NIOSH 2013).

There is limited epidemiological evidence with respect to the potential of MWCNT to cause adverse health effects in humans. The association between occupational exposure to MWCNTs and effects on lung health and the immune system was assessed in a cross-sectional study consisting of MWCNT-exposed workers and matching, unexposed control population (Vlaanderen et al. 2017). Lung function and fractional exhaled nitric oxide (FENO) were assessed to evaluate lung health whereas complete blood cell counts and serum levels of 51 immune markers and three pneumoproteins were determined to assess the effect of exposure to MWCNTs on the immune system. Regression analysis of the data obtained demonstrated significant correlation between exposure to MWCNT and increase in the serum levels of specific immune markers, such as the C-C motif ligand, basic fibroblast growth factor, and soluble interleukin-1 (IL-1) receptor II. There were also significant differences in the blood cell counts and FENO between the MWCNT-exposed and control populations. However, no MWCNT exposure-associated difference in lung function was detected. These results, therefore, indicated the early effects of occupational exposure to MWCNT on lung health and the immune system. Significant associations between MWCNT exposure and sputum levels of interleukins IL-1β, IL-6, tumor necrosis factor-α (TNF-α), and Krebs von den Lungen-6 were reported by Fatkhutdinova et al. (2016) in a cross-sectional study that included MWCNT-exposed workers and an unexposed, control population. Genome-wide differential expression analysis revealed changes in the expression profiles of mRNA and noncoding RNA in the blood samples obtained from a population that consisted of workers ($n = 8$) who were exposed to MWCNT for at least 6 months and for nonexposed controls ($n = 7$) (Shvedova et al. 2016). In a Korean population consisting of MWCNT-exposed workers and matching control subjects, Lee et al. (2015) determined lung function, hematology, and blood chemistry parameters. Additionally, markers of oxidative stress were determined in the exhaled breath condensate (EBC) collected from the subjects. No MWCNT exposure-related changes were detected in lung function, hematology, and blood chemistry parameters among the subjects. However, significant increases in markers of oxidative stress, such as malondialdehyde, n-hexanal, and 4-hydroxy-2-hexenal, were detected in the EBC of the MWCNT-exposed subjects that correlated with their blood molybdenum concentration, suggesting that the oxidative stress markers in EBC and blood molybdenum concentration may be useful biomarkers of MWCNT exposure.

Despite the availability of limited epidemiological data demonstrating the potential human health effects associated with occupational exposure to MWCNT, substantial evidence with respect to the pulmonary toxicity of MWCNT has been obtained from the results of studies conducted by employing animal models of human lung toxicity. These studies have demonstrated the induction of inflammation, oxidative stress, granuloma formation, fibrosis, tissue injury, genotoxicity, mesothelioma, tumorigenesis, and changes in the gene expression profile in the lungs in response to pulmonary exposure to MWCNTs as described in detail in the following sections. A vast majority of the *in vivo* studies that investigated the pulmonary toxicity caused by MWCNTs were conducted by employing either a mouse or rat as the animal model. Furthermore, in both these species, intratracheal instillation, pharyngeal aspiration, and inhalation were employed as exposure techniques to investigate pulmonary toxicity.

Lung Injury

Lung injury resulting from pulmonary exposure to MWCNTs has been determined either by assaying the lactate dehydrogenase (LDH) activity in the bronchial alveolar lavage (BAL) fluid or by conducting a histopathological analysis of the lungs. A significant elevation in the BAL level of LDH activity has been detected in the lungs in response to pulmonary exposure to toxic agents (Sager et al. 2020; Sellamuthu et al. 2011a), which has been considered as an indicator of lung toxicity. Significantly elevated BAL LDH activity in the lungs of MWCNT-exposed rats and mice has been reported (Aiso et al. 2010; Kasai et al. 2015; Porter et al. 2010; Sager et al. 2022), suggesting their potential for lung injury.

Lung histopathological changes, compared with appropriate control lungs, are most widely accepted as indicators of lung injury, resulting from pulmonary exposure to MWCNTs. Histological changes, suggestive of potential lung injury in MWCNT-exposed lungs, have been reported in most murine studies that investigated the pulmonary toxicity of MWCNTs. Despite the differences in the severity of the lung injury, depending on the dose and duration of the MWCNT exposure and the post-exposure time-period when histopathological analysis was performed (see details below), histopathological changes indicating lung injury have been detected in almost all toxicity studies reported so far.

Pharyngeal aspiration of MWCNT-7, one form of MWCNT, manufactured by Mitsui & Company (Tokyo, Japan) in mice at doses ranging from 10 to 80 μg and analysis of the lung sections obtained at post-exposure time intervals of 1, 7, 28, and 56 days revealed histopathological changes, including inflammation, fibrosis, and cellular proliferation (Porter et al. 2010). The MWCNT-7-induced pulmonary inflammation in mice was detected mainly in the interstitium but sometimes extending into the lumen at the bronchoalveolar junction, resulting in airway obstruction. Hypertrophy and hyperplasia of the bronchiolar and alveolar epithelial cells were also induced by MWCNT-7 in mice. In addition, granulomatous and fibrotic changes were detected in the lungs of MWCNT-7-administered mice. Penetration of the pleura by MWCNT particles and pleural inflammation, especially at the 56-day post-exposure time interval in mice administered the highest dose of 80 μg MWCNT-7, were also detected. Similar histological changes, indicative of lung injury, were also detected in mice that were exposed to MWCNT-7 by whole-body inhalation (Fraser et al. 2021; Mercer et al. 2013; Porter et al. 2013).

Whole-body inhalation exposure of rats to MWCNT-7 (1.25, 2.5, 5, or 10 mg/m^3, 6 hours/day, 3 days) resulted in dose-dependent histological changes in lungs that mainly consisted of the accumulation of inflammatory cells (alveolar macrophages [AMs] and neutrophils), thickening of the alveolar wall, and granuloma formation (Sager et al. 2022). Similar, dose-dependent histopathological changes were detected in male and female rats exposed by whole-body inhalation to MWCNT-7 (0.2, 1, and 5 mg/m^3 for 6 h/day, 5 days/week, for 13 weeks) (Kasai et al. 2015). Dose-dependent infiltration of inflammatory cells, type II cell hyperplasia, granuloma formation, and fibrosis with alveolar wall thickening were detected in the lungs of rats administered MWCNT-7 by intratracheal instillation (Aiso et al. 2010).

Inflammation

Induction of inflammation, in response to the presence of a toxic material in an organ or tissue, is characterized by the recruitment of leukocytes to the site where the toxic material is present and the release of inflammatory mediators, such as pro-inflammatory cytokines/chemokines. Induction

of inflammation is generally considered as a protective response to fight against the potential harmful effects of toxic materials. However, excessive inflammatory response may result in tissue injury. The MWCNT entering the lungs are phagocytosed, mainly by the AMs, for their elimination from the lungs. However, due to their elongated, fibrous structure and poor solubility, MWCNTs are not efficiently phagocytosed and eliminated, resulting in their prolonged persistence in the lungs. For example, the retention halftimes, an index of biopersistence, of MWCNTs were 151, 350, 318, and 375 days, respectively, following a 13-week, sub-chronic inhalation exposure of rats to MWCNTs at 0.1, 0.4, 1.5, and 6 mg/m^3 (Pauluhn 2010). The deposition of MWCNT particles in the alveoli and their interaction with the AMs result in the activation of the macrophages and the release of signaling molecules, including pro-inflammatory cytokines/chemokines. The pro-inflammatory cytokines/chemokines released by the AMs function as chemoattractants and facilitate the recruitment of additional inflammatory phagocytes, mainly polymorphonuclear neutrophils (PMNs), from blood to the lungs. This, in turn, results in the release of additional pro-inflammatory cytokines/chemokines culminating in the induction of excess inflammation. Induction of persistent or chronic inflammation is characteristic to MWCNTs due to their relatively long half-time and biopersistence and has been recognized as a major mechanism underlying the MWCNT-induced lung toxicity (Ellinger-Ziegelbauer and Pauluhn 2009; Ma-Hock et al. 2009; Porter et al. 2010, 2013; Hamilton et al. 2013; Poulsen et al. 2015, 2016; Dong and Ma 2016a; Kasai et al. 2015; Fujita et al. 2016; Gate et al. 2019; Sager et al. 2022).

Regardless of the technique employed to administer MWCNTs (intratracheal instillation, oropharyngeal aspiration, and nose-only or whole-body inhalation exposure), pulmonary exposure of mice and rats to the ENM, in a majority of the studies reported so far, resulted in a dose-dependent pulmonary inflammatory response. The number of PMNs present in the BAL samples was determined as the index of lung inflammation at 1, 7, 28, and 56 days, following a single oropharyngeal aspiration of 0, 10, 20, 40, or 80 μg MWCNT-7 in 7-week-old male C57BL/6 mice (Porter et al. 2010). The MWCNT-7 dose-dependent increase in the number of PMNs detected in the BAL of the mice peaked at the 7-day post-exposure period and returned to the control level by the 56-day post-exposure period with the exception of the group of mice that received the highest dose of 80 μg MWCNT-7. In a recent dose–response study conducted in our laboratory (Sager et al. 2022), Fisher 344 rats were exposed by whole-body inhalation to air (control) or an aerosol containing MWCNT-7 particles, ranging in concentrations from 1.25 to 10 mg/m^3 for 6 hours/day for 3 days. Analysis of the bronchoalveolar lavage (BAL) cells at the end of the exposure period revealed significant and MWCNT-7 dose-dependent increases in the number of total BAL cells, AMs, and PMNs in the exposed rats, compared with the controls. The induction of lung inflammation in MWCNT-7-exposed rats, revealed by the increase in the number of BAL cells, was further supported by the significant and MWCNT-7 dose-dependent elevations in BAL levels of multiple cytokines (IL-1β, IL-10, IL-13, IL-16, IL-18), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), and TNF-α (Sager et al. 2022). The inflammatory response to pulmonary exposure to a long and thick and a short and thin form of MWCNTs was determined in the lungs of Sprague-Dawley rats (Gate et al. 2019). The rats were exposed to two forms of MWCNT either by a single intratracheal instillation (180 and 540 μg) or by nose-only inhalation (0.5 and 1.5 mg/m^3, 2 × 3 hours/day, 5 days/week for 4 weeks). The number of PMNs infiltered into the lungs was determined at 1 and 28 days following the intratracheal or 3, 30, 90, and 180 days following inhalation exposure, as an index of MWCNT-induced lung inflammation. The dose-dependent inflammatory response detected in MWCNT-exposed rat lungs was similar, irrespective of the type of MWCNT or the method of administration, when the deposited dose was considered. This, in agreement with the findings of Poulsen et al. (2016), suggested that the deposited surface area of

MWCNT is an excellent predictor of pulmonary neutrophil infiltration, a hallmark of lung inflammation in response to pulmonary exposure to toxic agents, including ENMs. Induction of lung inflammation, a major response to the pulmonary exposure to MWCNTs, has also been demonstrated in several other studies that employed mouse (Dong and Ma 2016a; Hamilton et al. 2013; Porter et al. 2013; Poulsen et al. 2015) or rat (Ellinger-Ziegelbauer and Pauluhn 2009; Fujita et al. 2016; Kasai et al. 2015; Ma-Hock et al. 2009) as the lung toxicity model.

Oxidative Stress

Under normal physiological conditions, reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, and hydroxyl radical – the by-products of cellular respiration and metabolic activities – play an important role in maintaining tissue homeostasis. The biological system is equipped with protective mechanisms capable of detoxifying toxic ROS. However, excessive generation of ROS may exceed the capability of the biological system to detoxify them. This may result in the accumulation of toxic ROS in tissues and organs that, in turn, may lead to oxidative stress and damage to cellular components, including macromolecules, such as lipids, DNA, and proteins. The oxidative stress resulting from excessive generation and tissue accumulation of ROS has been identified as a contributing factor in several chronic and potentially fatal diseases including cancer (Hayes et al. 2020).

Induction of oxidative stress has been identified as a major mechanism underlying the toxicity of inhaled particles such as crystalline silica (Sager et al. 2020) and asbestos (Pietrofesa et al. 2016) with sizes larger than those of ENMs. Multiple mechanisms have been identified to contribute to particle-induced generation of reactive oxidants and the resulting oxidative stress. The particles may possess reactive oxygen molecules on their surface, which, following their entry into the lungs, may directly result in the induction of oxidative stress (Vallyathan et al. 1988). Some of the inhaled particles may contain trace metals, such as iron as contaminants that may generate ROS through Fenton reaction, resulting in the induction of oxidative stress (Diabate et al. 2002; Voelkel et al. 2003). Another mechanism involved in the particle-induced generation of ROS is through their interaction with lung phagocytes, mainly AMs. The AMs phagocytose the inhaled particles deposited in the alveoli to eliminate them from the lungs, preventing their accumulation and the resulting toxicity. However, due to physical characteristics such as their elongated structure and insolubility, MWCNTs are not efficiently engulfed and removed by AMs. This may result in frustrated phagocytosis with eventual damage to the AMs and the release of the engulfed particles along with reactive and toxic intermediates, including ROS, potentially resulting in oxidative stress and tissue injury. Metal contaminants present in some preparations of MWCNTs have been found to be responsible for the induction of oxidative stress (Pulskamp et al. 2007). Some preparations of MWCNT-7, on the other hand, do not contain adequate quantities of metal contaminants to induce oxidative stress (Porter et al. 2010). Furthermore, the inability of CNTs to directly generate ROS in a cell-free system has been demonstrated (Fenoglio et al. 2006; Porter et al. 2010). In the absence of metal contaminants, the direct interaction of MWCNTs with AMs has been suggested as the mechanism responsible for the generation of ROS in the lungs (Sager et al. 2022).

In the study reported by Sager et al. (2022), rats were exposed by whole-body inhalation to air or an aerosol that contained increasing concentrations of MWCNT-7 for three days. The lung phagocytes obtained, following euthanasia, from the control and MWCNT-7-exposed rats were used in a chemiluminescence assay to determine the generation of reactive oxidants either by the entire population of the phagocytes or the AMs alone present in the lungs. The results obtained from the study (Sager et al. 2022) demonstrated a significant ($p < 0.05$) and dose-dependent generation of

reactive oxidants in response to inhalation exposure to MWCNT-7 in rats. Similar to these results, a dose-dependent generation of reactive oxidants and their potential role in the lung toxicity induced in response to pulmonary exposure to MWCNT-7 have been reported by Kato et al. (2013).

The importance of reactive oxidant generation and the resulting oxidative stress in lung toxicity in response to exposure to MWCNT-7 has been demonstrated by Dong and Ma (2016b) by employing a knock-out mouse model that does not express the nuclear factor erythroid-2-related factor 2 (Nrf2) gene and matching wild-type mice. The Nrf2 is a redox-sensitive transcription factor that regulates the expression of key genes involved in protecting the biological system through the detoxification of toxic reactive oxidants (Kensler et al. 2007). The Nrf2 knock-out ($Nrf2^{-/-}$) and wild-type ($Nrf2^{+/+}$) mice were exposed to MWCNT-7 at doses of 0, 5, 20, and 40 μg by oropharyngeal aspiration and the AMs obtained from the mice were analyzed for the presence of ROS at post-exposure time intervals of 1, 3, 7, and 14 days. The number of ROS-producing AMs and the intensity of ROS staining in them, in response to the pharyngeal aspiration of MWCNT-7, were significantly higher in $Nrf2^{-/-}$ mice compared with $Nrf2^{+/+}$ mice. Similar differences were noticed in the parameters employed to determine oxidative damage to DNA and lipids between $Nrf2^{-/-}$ and $Nrf2^{+/+}$ mice, in response to their exposure to MWCNT-7. Collectively, these results demonstrated the induction of oxidative stress in response to pulmonary exposure to MWCNT-7 and the role of Nrf2 in regulating the MWCNT-7-induced oxidative stress.

Fibrosis

The high aspect ratio, rigidity, insolubility, and fibrous structure of MWCNT, like that of asbestos, have raised concerns about their potential, if inhaled, to cause lung fibrosis (Donaldson et al. 2006). There is compelling evidence in the literature suggesting the fibrogenic potential of MWCNT-7 with significant resemblance to that of a foreign substance-induced fibrotic response that is initiated with an acute phase response followed by chronic, interstitial fibrosis with or without granuloma formation (Mercer et al. 2013; Porter et al. 2010, 2013). In a dose–response and time-course study reported by Dong et al. (2015), MWCNT-7 particles were administered to C57BL/6J mice by pharyngeal aspiration at concentrations of 0, 5, 20, and 40 μg. The lungs obtained from the control and MWCNT-7 exposed mice were analyzed for histological changes, indicative of fibrosis, at post-exposure time intervals of 1, 3, 7, and 14 days. Rapid and dose-dependent appearance of collagen fibers in the lungs of the MWCNT-7 exposed mice were detected by Masson's trichrome and Picrosirius staining. Fibrotic foci, especially near MWCNT-7 deposited in the lungs, were also detected. The rapid appearance of lung fibrosis was detected in mouse lungs as early as one day following the MWCNT-7 exposure and persisted throughout the observation period. A similar lung fibrotic response was noticed by Porter et al. (2010) in another mouse study in which the animals were exposed to MWCNT-7 by whole-body inhalation (10 mg/m^3, 5 hours/day) for 2, 4, 8, or 12 days and their lungs were assessed at one day post exposure for histological changes indicative of fibrosis. Similar to the mouse studies, pulmonary exposure of rats to MWCNT-7 also resulted in lung fibrosis. In a dose–response study (Sager et al. 2022) in which rats were exposed by whole-body inhalation to MWCNT-7 at concentrations of 1.25, 2.5, 5, and 10 mg/m^3, 6 hours/day for 3 days, thickening of the alveolar wall and presence of collagen fibers, indicative of lung fibrosis, were detected at the end of the exposure period. In another rat whole-body inhalation study (Kasai et al. 2015), male and female rats were exposed to aerosols containing MWCNT-7 particles at concentrations of 0, 0.2, 1, and 5 mg/m^3 for 13 weeks. Increased accumulation of collagen fibers, in association with granulomatous changes, were detected in the lungs, which further supported

the induction of lung fibrosis by MWCNT-7 in rats. Lung fibrosis was also detected in rats in response to intratracheal instillation of MWCNT particles (Muller et al. 2005).

A close relationship between the induction of lung inflammation and fibrosis in response to pulmonary exposure to MWCNT-7 has been reported in many studies (Dong and Ma 2016a; Dong et al. 2015; Porter et al. 2010). For example, the appearance of collagen fibers, a hallmark of fibrosis, is most abundantly detected in granulomas appearing in MWCNT-7 exposed lungs (Porter et al. 2013; Sager et al. 2022). By employing a mouse model for allergic asthma, Ryman-Rasmussen et al. (2009) have demonstrated the role of preexisting inflammation in MWCNT-induced lung fibrosis. Control and ovalbumin-sensitized mice were exposed to air or MWCNT (100 mg/m^3) by inhalation for 6 hours; at 1 and 14 days following the termination of the exposures, lung injury, inflammation, and fibrosis were determined. At 14 days post exposure, lung fibrosis was detectable only in mice that received both ovalbumin and MWCNT. Ovalbumin sensitization or MWCNT exposure alone did not result in lung fibrosis suggesting that the existence of inflammation is a requirement for MWCNT-induced lung fibrosis. It is generally accepted that a biphasic pathologic development of lung fibrosis that includes transition from an initial acute inflammatory response to interstitial fibrosis is characteristic of MWCNT-induced lung fibrosis.

Further insight into the mechanisms underlying MWCNT-7-induced lung fibrosis is presented in a series of papers published by Dong and Ma (2016b, 2017a, 2017b). Transgenic mice models were employed in their studies to demonstrate the role of specific genes and the mechanisms involved in MWCNT-7-induced lung fibrosis. In one of the studies (Dong and Ma 2016b), MWCNT-7 was administered in Nrf2$^{+/+}$ and Nrf2$^{-/-}$ mice by pharyngeal aspiration at doses of 0, 5, 20, and 40 μg, and the resulting lung toxicity, including induction of oxidative stress, inflammation, and fibrosis, was detected on 1, 3, 7, and 14 days following the exposure. Infiltration of phagocytes, especially PMNs, and the appearance of inflammatory and fibrotic foci were detected in all mice administered MWCNT-7. Similarly, generation of fluorescence due to the ROS-mediated oxidation of the fluorophore, dihydroethidium, was detected in the AMs obtained from the lungs of MWCNT-7-exposed mice. However, lung inflammation, fibrosis, and oxidative stress, in response to MWCNT-7 exposure, were more in MWCNT-7-administered Nrf2$^{-/-}$ mice compared with Nrf2$^{+/+}$ mice. These results demonstrated the protective role of the Nrf2 gene in MWCNT-7-induced lung toxicity. Furthermore, the findings suggested the interplay between Nrf2 and oxidative stress, inflammation, and fibrosis, determining the pathological outcomes of MWCNT-7 exposure.

The role of the gene, tissue inhibitor of metalloproteinase 1 (Timp1), in MWCNT-7-induced lung fibrosis and the underlying mechanisms were investigated by Dong and Ma (2017b). Timp1 wild-type (Timp1$^{+/+}$) and knock-out (Timp1$^{-/-}$) mice were exposed to 0 or 40 μg MWCNT-7 by pharyngeal aspiration and at post-exposure time periods of 1, 3, 7, and 14 days, and the lungs obtained from the euthanized mice were analyzed for fibrotic changes. Increased lung deposition of matrix proteins, collagen I, and fibronectin was detected in MWCNT-7-exposed mice, compared with the vehicle-only-administered mice, regardless of their genotype. Similarly, recruitment, activation, and proliferation of fibroblasts were higher in MWCNT-7-exposed mouse lungs. Differentiation of fibroblasts to myofibroblasts, an important step in fibrosis development (Barnes and Gorin 2011), was also higher in MWCNT-7-exposed mouse lungs. All fibrosis-related cellular events viz. recruitment, activation, and proliferation of fibroblasts, and their differentiation to myofibroblasts, taking place in response to exposure to MWCNT-7 were more in Timp1 wild-type mice compared with knock-out mice. These results, therefore, suggested the important role played by the Timp1 gene in MWCNT-7-induced lung fibrosis. Furthermore, it was demonstrated that the modulation of the MWCNT-7-induced pulmonary fibrotic response by Timp1 was mediated through the Timp1/CD63/integrin β1 axis and extracellular-signal-regulated kinase signaling.

Osteopontin (OPN; secreted phosphoprotein 1 or SPP1) is a gene that has been found overexpressed in the blood of workers (Fatkhutdinova et al. 2016) and the lungs of rats (Sager et al. 2022) in response to MWCNT-7 exposure. Osteopontin functions both as a cytokine and an extracellular matrix (ECM) protein and has been implicated in functions relevant to fibrosis, such as infiltration of inflammatory cells and tissue remodeling (Rittling and Singh 2015). In addition, OPN is highly overexpressed during wound healing (Liaw et al. 1998) and significant induction of OPN has been reported in animal models of fibrosis (Dong et al. 2016; Sabo-Attwood et al. 2011). The role of OPN in MWCNT-7-induced lung fibrosis was investigated by Dong and Ma (2017a) by employing OPN knock-out ($OPN^{-/-}$) and wild-type ($OPN^{+/+}$) mice. A single dose of 40 μg MWCNT-7 that is known to result in lung fibrosis in mouse was administered in $OPN^{-/-}$ and $OPN^{+/+}$ mice by pharyngeal aspiration. The lungs obtained from the vehicle and MWCNT-7-administered mice were analyzed for fibrosis at 1, 3, 7, 14, 28, and 56 days following exposure. Both OPN mRNA and protein were significantly overexpressed, at all post-exposure time intervals analyzed, in MWCNT-7-administered $OPN^{+/+}$ mice. Fibrotic lesions (appearance of fibrotic foci and detection of collagen fibers by Masson's trichrome staining) assessed in the lungs of MWCNT-7-exposed $OPN^{+/+}$ mice exhibited a biphasic response consisting of an early rapid phase that peaked on day 7 and resolved by day 14. The early phase was followed by a chronic phase, fully developed on day 28 and lasted until day 56. Compared with MWCNT-7-exposed $OPN^{+/+}$ mice, the lung fibrotic response detected in the $OPN^{-/-}$ counterparts was much milder. Expressions of ECM marker proteins and transformation of fibroblasts to myofibroblasts were much higher in the lungs of MWCNT-7-exposed $OPN^{+/+}$ mice, compared with the corresponding $OPN^{-/-}$ mice. The profibrotic role of OPN in the MWCNT-7-exposed mouse lungs was mediated through the activation of Smad-dependent TGF-β1 signaling.

Mesothelioma

The similarity of MWCNT to asbestos with regard to features, such as high aspect ratio, long and needle-like shape, rigidity, and biopersistence, have raised serious concerns regarding their potential to cause mesothelioma (Donaldson et al. 2006). Mesothelioma is a toxicological response unique to fibrous particles (Craighead et al. 1982). According to the fiber-pathogenicity paradigm (Donaldson and Tran 2004), exposure to biopersistent fibers with a diameter less than 3 μm and length more than 10–20 μm is likely to result in mesothelioma. Intraperitoneal administration of MWCNT in mice (Takagi et al. 2008, 2012) and rats (Rittinghausen et al. 2014) resulted in the appearance of mesothelioma. Similar results were obtained following intrascrotal administration of MWCNT in rats (Sakamoto et al. 2009). Donaldson et al. (2010) hypothesized that long fibrous particles deposited in the lungs are retained within the parietal pleura and initiate pathological events, such as the induction of inflammation and granuloma formation resulting in mesothelioma. This was based on the earlier demonstration that intraperitoneal administration of MWCNT resulted in asbestos-like pathogenicity in mice (Poland et al. 2008). Additional evidence for the length of the fibrous particles being the main determinant in the induction of mesothelioma is derived from the observation that pulmonary deposition of long but not short crocidolite particles resulted in pleural fibrosis (Adamson et al. 1993) and proliferative responses (Adamson et al. 1994) in mice. Similarly, intraperitoneal administration of longer fiber particles, compared with shorter particles, resulted in greater inflammatory and granuloma-generating response in mice (Donaldson et al. 1989; Goodglick and Kane 1990). It was suggested that the failure of the AMs to completely enclose or engulf the longer fibrous particles resulting in frustrated phagocytosis is

likely responsible for their accumulation and initiation of pathological conditions in mesothelial cells culminating in the development of mesothelioma (Poland et al. 2008).

Experimental evidence for the retention of long MWCNT particles in the parietal pleura as a mechanism responsible for the induction of mesothelioma, resulting from exposure to MWCNT was obtained from a study conducted by Murphy et al. (2011). In the study, an intrapleural instillation method was employed to administer particles of different lengths directly into the pleura of mice through the chest wall. The particles viz. long and short MWCNT, long and short asbestos fibers, and nanoparticle carbon black, were administered in different groups of female C57BL/6 mice. At post-exposure time periods consisting of 24 hours, 7 days, 4 weeks, 12 weeks, and 24 weeks, the mice were euthanized, and pleural lavage was performed to assess pleural inflammatory response. Simultaneously, the parietal pleura of the mice was assessed for histological changes and retention of the administered particles. Pleural inflammation, characterized by the infiltration of granulocytes, was prominent in mice administered long, fibrous particles. Parietal pleura showed histological changes consistent with the inflammatory response detected in mice administered the particles. Cellular proliferation in the mesothelium of the parietal pleura was also detected only in mice administered long, fibrous particles. Furthermore, retention of the long, fibrous particles was detected in the pleural space of mice. The shorter particles were found deposited in the lymph nodes of mice, suggesting their elimination from the pleural space. Collectively, the results provided evidence for the length of the MWCNT particles as the major determinant of their potential to cause mesothelioma in the mouse model employed in the study. While the shorter MWCNT particles administered in the pleural cavity egressed through the stomata, the longer particles failed to be cleared from the pleura through the stomata. This resulted in the retention of longer MWCNT particles in the pleura of mice, leading to inflammatory and granulomatous responses characteristic of mesothelioma.

Lung Cancer

To date, there are no epidemiological data to support that human exposure to MWCNT results in lung cancer. Nevertheless, the International Agency for Research on Cancer (IARC) has classified MWCNT-7, one form of MWCNT that was employed in multiple animal toxicity studies (Porter et al. 2010; Dong and Ma 2016a, 2016b, 2017a, 2016b; Dong et al. 2015, 2016; Kasai et al. 2015; Umeda et al. 2013; Sager et al. 2022), as a Group 2B carcinogen (possibly carcinogenic to human) (IARC 2017). The classification of MWCNT-7 as a Group 2B carcinogen is based on the positive results obtained from mouse and rat carcinogenicity studies. In the mouse carcinogenicity study, Sargent et al. (2014) administered either the vehicle or a single intraperitoneal dose (10 μg/g body weight) of the tumor initiator, methylcholanthrene, in mice, which was followed by their whole-body inhalation exposure to air or MWCNT-7 (Mitsui and Company, Tokyo, Japan) at a concentration of 5 mg/m^3 for 5 hours/day for 15 days. At 17 months following the MWCNT-7 exposure, incidence of tumor formation was determined in the lungs obtained from the mice that received the vehicle (control), MCA alone, MWCNT-7 alone, or MCA plus MWCNT-7. The tumor incidence in the lungs of the mice that received either MCA or MWCNT-7 alone was not significantly different compared with the mice in the control group. On the other hand, a significantly higher incidence of tumorigenesis was detected in the lungs of the mice that received MCA plus MWCNT-7, compared with those received either MCA or MWCNT-7 alone. These results, therefore, suggested that MWCNT-7 is a tumor promoter in a mouse two-stage carcinogenesis model.

The lung carcinogenicity of MWCNT-7 was investigated in both sexes of the F344 rats by Kasai et al. (2016). The rats were chronically exposed by inhalation to air or aerosols containing MWCNT-7 at concentrations of 0.02, 0.2, or 2 mg/m^3, 6 hours/day, 5 days/week for 104 weeks and the incidence of tumorigenesis was recorded. A significant and MWCNT-7 dose-dependent increase in the incidence of bronchioloalveolar carcinoma, adenosquamous carcinoma, adenocarcinoma, and squamous cell carcinoma was detected in both the sexes of the rats. However, the incidence of lung tumorigenesis was more in male rats, suggesting a gender difference in the tumorigenic potential of MWCNT-7. For example, a statistically significant increase in lung tumor formation was detected in male and not in female rats that belonged to the 0.2 mg/m^3 exposure group. No tumor formation was detected in any organs or tissues other than the lungs despite the translocation of the administered MWCNT-7 particles into the pleural and peritoneal cavities in rats. The results of the rat carcinogenesis study by Kasai et al. (2016) suggested that MWCNT-7 is a complete carcinogen in rats. The results may also suggest that the dose and/or the duration of MWCNT-7 exposure employed in the mouse two-stage carcinogenesis study conducted by Sargent et al. (2014) was not sufficient to cause lung tumor formation in mice that were exposed to MWCNT-7 alone. The decreased sensitivity of mice, compared with rats, to develop lung tumors following pulmonary exposure to particles (Mauderly 1997) may also account for the absence of lung tumors detected in mice exposed to MWCNT-7 alone.

Contradictory results, with respect to the lung tumorigenic potential of another form of MWCNT, designated as MWCNT-N (Nikkiso, Tokyo, Japan), have been reported in rats. In a study conducted by Suzui et al. (2016), rats were intratracheally administered MWCNT-N at doses of 0 or 125 μg/rat, 8 times over a period of 2 weeks. Compared with the control rats, the incidence of lung tumors, at 109 weeks following MWCNT-N administration, was significantly higher in the exposed rats. In contrast to these findings, no lung tumors were detected in rats at 2 years following the intratracheal administration of MWCNT-N at doses of 0.67 or 3.3 mg/kg body weight (Nakanishi et al. 2015).

Genotoxicity

Genotoxicity has attracted much attention as a major mechanism responsible for the carcinogenicity of toxic agents. In a study reported by Kato et al. (2013), the genotoxicity potential of MWCNT-7 was demonstrated in *in vitro* cell culture and *in vivo* mouse models by employing a battery of tests. Exposure of human lung carcinoma A549 cells or Chinese hamster ovary AA8 cells to MWCNT-7 resulted in a dose-dependent increase in the frequency of the sister chromatid exchange or micronuclei, respectively, suggesting the *in vitro* genotoxicity potential of MWCNT-7. These results were further supported by the positive results obtained in mice in response to their exposure to MWCNT-7. Results of the comet assay revealed a significant increase in the incidence of DNA damage in the lungs of ICR mice intratracheally instilled with a single dose (0.05 or 0.2 mg/mouse) of MWCNT-7. A similar increase in the mutation frequency of the *gpt* gene, further supporting the genotoxicity of MWCNT-7, was detected in the lungs of the *gpt* delta transgenic mouse. Furthermore, dose-dependent increases in the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine and heptanone etheno-deoxyribonucleosides detected in the lungs of the MWCNT-7-exposed mice, prompted the authors to suggest the involvement of oxidative stress in the genotoxicity resulting from the exposure to MWCNT-7. Similar evidence of genotoxicity has been reported based on the observation of DNA strand breaks in mouse lungs in response to their pulmonary exposure to MWCNT (Catalan et al. 2016).

Further evidence for the genotoxicity of MWCNT was obtained from the results of a mouse study conducted by Poulsen et al. (2016). The genotoxicity of 10 commercial MWCNT samples that differ in their morphology and physical and chemical characteristics was determined in C57BL/6J mice. MWCNTs used in the study were either pristine or surface modified and exhibited differences in their morphology, chemical composition, surface area, and functionalization status. Various strengths of MWCNTs were intratracheally administered in mouse lungs at doses of 0, 6, 18, or 54 μg/mouse. At post-exposure time intervals of 1, 28, or 92 days, DNA strand breaks – indicators of genotoxicity – were determined by comet assay in the lungs of the control and MWCNT-administered mice. Furthermore, the authors, by multiple regression analysis of the data, determined the relationship, if any, between the physicochemical properties of the MWCNT samples and their genotoxicity potential. The authors found that pulmonary exposure to MWCNTs in mice resulted in lung genotoxicity and the Brunauer–Emmett–Teller (BET) surface area of MWCNT particles that correlates well and inversely with the diameter was a good predictor of MWCNT-induced genotoxicity. A lower BET surface area or a corresponding larger diameter of the MWCNT particles was associated with increased genotoxicity in mouse lungs. Similar findings were also reported by Fraser et al. (2020).

Whether exposure to MWCNT-7 results in changes in the chromosome number, a key genotoxic event potentially responsible for carcinogenesis, was investigated by employing immortalized human bronchial epithelial cells (BEAS-2B) and primary small airway epithelial cells (SAEC) (Siegrist et al. 2014). Exposure of the cultured BEAS-2B and SAEC cells to occupationally relevant doses of MWCNT-7 resulted in changes in chromosome number, suggesting induction of aneuploidy as a potential genotoxic mechanism involved in MWCNT-7-induced carcinogenesis. The chromosomal aberrations induced by MWCNT-7 exposure were attributed to the disruption of centrosomes and interference with the mitotic spindle apparatus in association with the cell cycle block. Furthermore, the genetically damaged, aneuploid BEAS-2B cells exhibited increased colony formation, a characteristic of the transformed phenotype, further supporting the induction of aneuploidy as a potential mechanism underlying the neoplastic potential of MWCNT-7.

Contrary to the above reports, Ema et al. (2013) did not find any evidence to support the genotoxicity of MWCNTs in rats. Male Crl/CD(SD) rats were intratracheally administered MWCNTs as a single dose (0.2 or 1 mg/kg body weight) or repeated doses of 0.04 or 0.2 mg/kg body weight, once a week for 5 weeks. The control and MWCNT-administered rats were euthanized (3 or 24 hours following a single instillation or 3 hours following the previous instillation in the repeatedly administered groups) and the DNA obtained from their lungs was analyzed using the comet assay for DNA damage. The findings of the study showed that MWCNT samples employed in the study, under exposure conditions, did not result in genotoxicity in rats. It is worth mentioning that significant lung inflammation was detected in MWCNT-administered rats, under the same exposure conditions, compared with the corresponding control rats. Similar to the findings of Ema et al. (2013), the absence of *gpt* gene mutation, an indicator of genotoxicity, has been reported in the lungs and bone marrow of F344 *gpt* delta rats that were intratracheally administered a single dose of MWCNT (Horibata et al. 2017).

Toxicogenomics of ENMs

Over the past 30 years, the toxicology field has undergone a major transformation. This was facilitated mainly by the realization that determination of global changes in cellular molecules, collectively referred to as the "-ome," may provide broader information regarding the toxicity of

xenobiotics than the traditional approaches employed in toxicity assessment, such as the histological, biochemical, and physiological changes indicative of toxicity. Specifically, the "-ome" refers to the whole array of molecules, such as DNA, RNA, proteins, and lipids, present in the cells and metabolites generated from xenobiotics. This resulted in the emergence of a new branch in toxicology, referred to as toxicogenomics. The US National Research Council (NRC) defines toxicogenomics as "combin(ing) toxicology with information-dense genomic technologies to integrate toxicant-specific alterations in gene, protein, and metabolite expression patterns with phenotypic response of cells, tissues, and organisms" (NRC 2007). Toxicogenomics facilitates the interrogation of cellular response to a xenobiotic exposure at the global, molecular level (Alexander-Dann et al. 2018). The advances in molecular biology, computer science, mathematics, bioinformatics, and the detection and analysis of metabolites played significant roles in the emergence and growth of the various branches in toxicogenomics such as genomics (study of the genome or DNA), transcriptomics (study of the transcriptome or transcripts), proteomics (study of the proteome or proteins), lipidomics (study of the lipidome or lipids), and metabolomics or metabonomics (study of the metabolome or metabolites or small molecules). Transcriptomics, by far, is more advanced than the other branches in toxicogenomics and has played a prominent role in studying the toxicity of xenobiotics including that of ENMs. Transcriptome profiling and bioinformatic analysis of the data obtained with crystalline silica and acetaminophen have facilitated the identification of sensitive biomarkers for the detection of target organ toxicity (Bushel et al. 2007; Sellamuthu et al. 2011a) as well as determining the molecular mechanisms underlying the toxicity (Sellamuthu et al. 2011b).

Transcriptomics – Technical Aspects

Transcriptomics, in the context of toxicogenomics, consists of two important steps. The first step involves the detection and identification of the transcripts that are differentially expressed in response to exposure to a toxic agent. Subsequently, during the second step, the functional relevance or significance of the differentially expressed genes (DEGs) in the ensuing biological response or toxicity is determined. Various techniques are currently available to determine the differential gene expression (DGE) profile in biological systems in response to their exposure to a toxic agent. The selection of a specific technique employed in DGE profiling depends on the coverage or the number of transcripts that the investigator(s) is(are) interested in determining. Real-time polymerase chain reaction (RT-PCR) is the method of choice when the objective is to determine the profile of a limited number of specific DEGs. On the other hand, if the objective is to determine the expression profile of all or majority of the transcripts present in the transcriptome, high-throughput techniques, such as microarray or next generation sequence (NGS) or, more specifically, RNA sequencing (RNA-seq) analysis, are preferred (Joseph 2017).

Among the three popular techniques currently employed to determine the DGE profiling, RT-PCR is the least complex one. It involves reverse transcription of the high-quality RNA isolated from the control and toxicant exposed biological samples to synthesize cDNAs followed by the DNA polymerase-catalyzed amplification, detection, and quantification of the cDNAs of interest that are annealed to specific oligonucleotide primers. The expression of the DEGs determined by RT-PCR is typically normalized to that of one or more housekeeping genes to obtain more reliable and acceptable data. Finally, the fold changes in expression of the transcript(s) between the toxicant exposed and the appropriate control sample is calculated to determine the DGE profile (Joseph 2017).

The microarray technique consists of sequence-specific hybridization of the transcripts (targets) prepared from a biological sample with hundreds or thousands of the oligonucleotides or cDNAs (probes) arrayed on the microarray platform. The various steps involved in microarray analysis of the DGE profile are the reverse transcription of mRNAs to synthesize cDNAs, their labeling with fluorescent dye(s) to prepare targets, sequence-specific hybridization of the labeled targets with oligonucleotides or cDNAs arrayed on the microarray, washing to remove nonspecific binding of targets with probes and the free labels potentially contributing to background signal, scanning the arrays to detect the fluorescence intensity associated with the hybridized target–probe complex, comparison of the signal intensity with appropriate control and xenobiotic-exposed samples to detect and quantify the DGE profile (Joseph 2017). Since its inception in the early 1990s (Bumgarner 2013), microarray technology has been employed in studying the toxicity of xenobiotics affecting virtually every organ or system in the body in a variety of species.

RNA-sequence (RNA-seq) analysis is the latest advancement in profiling the whole transcriptome for DGE. Since RNA-seq is based on *de novo* sequence detection, the technique can detect virtually all transcripts, including novel or previously uncharacterized transcripts that are present in the transcriptome. RNA-seq consists of multiple steps, such as the preparation of sequencing libraries from the control and xenobiotic-exposed biological samples, sequencing the libraries, and quantification of the transcripts to determine DGE between the control and xenobiotic-exposed samples (Figure 7.1). Since RNA molecules such as rRNA and globin mRNA that are present in very high abundance in biological samples are known to result in their overrepresentation during RNA-seq, complicating the detection of less abundant transcripts and their differential expression, it is important to selectively remove those abundant RNA species prior to or during library preparation. The next step in RNA-seq analysis is the reverse transcription of RNA to synthesize cDNA that are ligated to adapters that serve as barcodes to identify the cDNAs prepared from a sample. The adapter-ligated cDNA library samples are subsequently subjected to high-throughput sequencing, typically to a read depth of 10–30 million reads per sample, and the data is further analyzed to determine the differences in the gene expression profile between the toxicant exposed and appropriately matched control samples.

Analysis of the raw data generated by the techniques employed in DGE profiling is a critical step involved in the identification and quantification of DEGs. Data analysis can be simple or very

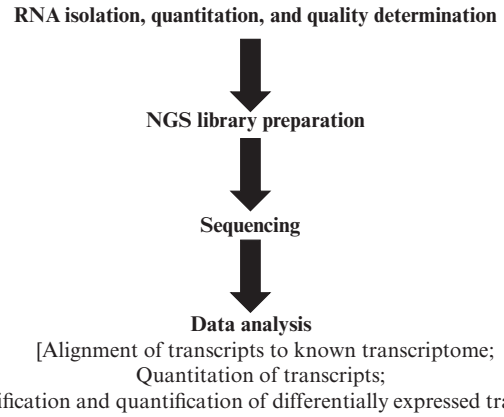

Figure 7.1 A schematic representation of the steps involved in differential gene expression profiling by RNA sequence analysis.

complex depending on the technique that is employed to generate gene expression data. Among the three currently employed techniques, data analysis is least complicated in the case of QRT-PCR and consists of normalization of the expression of gene(s) of interest (target gene[s]) to that of one or more housekeeping genes and calculating the fold changes in gene expression between the xenobiotic-exposed and matched control samples. Data analysis in the case of microarray is not as simple as QRT-PCR and typically consists of normalization of the hybridization signal intensities and calculating the fold changes in the expression of all of the detected genes (tens of thousands in the majority of cases) represented on the microarray between the sample sets. Data analysis in the case of RNA-seq, compared with QRT-PCR and microarray, is much more complex and requires significant expertise and computational resources (Stark et al. 2019). The first step in RNA-seq data analysis is alignment or mapping the sequence reads generated to a known transcriptome. Various alignment tools, such as TopHat (Kim et al. 2013), STAR (Dobin et al. 2013), or HISAT (Kim et al. 2015), are available and routinely employed. These tools facilitate the spliced alignment of the cDNA sequences generated by RNA-seq with the exon boundaries of the genes. The next step in data analysis is assignment of the sequences to genes or transcripts to determine their counts or abundance. Some of the common quantification tools employed in the RNA-seq analysis are RSEM (Li and Dewey 2011), CuffLinks (Trapnell et al. 2012), MMSeq (Turro et al. 2011), and HTSeq (Anders et al. 2015). Various factors, such as read depth, expression patterns of genes, GC content, and technical biases, are known to contribute to differences in the quantification of transcripts (Risso et al. 2011; Wagner et al. 2012) and, therefore, the data needs to be normalized and filtered to improve data quality. Quantile or median normalization (Bourgon et al. 2010) is commonly employed for this purpose. The final step in RNA-seq data analysis is differential expression modeling, determining the transcripts that may have changed in expression and quantifying the changes between the sample sets. Some of the tools commonly employed in this step of the RNA-seq analysis are edgeR (Robinson et al. 2010), DESeq2 (Love et al. 2014), and limma + voom (Law et al. 2014). Each of the steps involved in RNA-seq data analysis may significantly influence the DGE data output and, therefore, great care should be employed while analyzing the RNA-seq data to obtain biologically or toxicologically meaningful gene expression data.

Despite the challenges, especially those associated with data analysis, RNA-seq has several advantages compared with the microarray analysis in high-throughput DGE studies. Compared with microarray, RNA-seq is more sensitive and, therefore, capable of detecting transcripts that are present in very low abundance (Wang et al. 2014). Since microarray is based on the hybridization between the labeled transcripts (probes) expressed in the biological sample and the targets that are arrayed on the microarray platform, unknown or novel transcripts are not detected by the microarray technique. RNA-seq, on the other hand, involves *de novo* sequencing of all of the transcripts present in the biological specimen employed and, therefore, can detect novel transcripts and RNA species other than mRNA, such as miRNA, long noncoding RNA (lncRNA), and small nuclear RNA (snRNA) (Stark et al. 2019). RNA-seq, unlike microarray, can also detect splice variants of the transcripts. Despite being the newcomer in the field, RNA-seq is being increasingly utilized lately in DGE analysis in toxicogenomics (Joseph et al. 2021; Sager et al. 2020).

Toxicogenomics of MWCNTs – Animal Studies

The application of whole transcriptome profiling and bioinformatic analysis of the gene expression data to understand the molecular mechanisms of MWCNT-induced lung toxicity was recently investigated in our laboratory by employing a rat inhalation exposure and lung toxicity model

(Sager et al. 2022). Global gene expression profiles in the lung samples obtained from rats exposed to an aerosol containing increasing concentrations of MWCNT-7 and the corresponding air only exposed to controls was analyzed by NGS analysis. The number of the significantly differentially expressed genes (SDEGs) (fold change >1.5 and false discovery rate [$p < 0.05$]) detected in rat lungs (Sager et al. 2022), like the alterations in the toxicity parameters (Sager et al. 2022), exhibited a response to the cumulative dose of MWCNT-7 inhaled by the rats. Similarly, the fold changes in expressions of many of the SDEGs that are known to play a significant role in the pulmonary toxicity of MWCNT-7 were also dependent on the dose of the MWNCT-7 inhaled by the rats. The SDEGs identified in rat lungs by NGS analysis were used as input in the bioinformatic analysis to identify the biological functions and canonical pathways that were significantly enriched in response to inhalation exposure of the rats to MWCNT-7 and the resulting lung toxicity. The enrichment in the biological function and canonical pathway categories, similar to the alterations in the lung toxicity parameters, exhibited a dose response to the MWCNT-7 inhaled by the rats (Sager et al. 2022). Thus, the findings of the transcriptome analysis and those of lung toxicity determination suggested that the gene expression changes detected in rat lungs, in response to their exposure to MWCNT-7, were relevant to the lung toxicity induced by the inhaled MWCNT-7 in the rats.

Inhaled MWCNT particles, upon deposition in the lungs, are engulfed by phagocytes, mostly AMs, for their elimination from the lungs. The significant increase in the number of AMs detected in the MWCNT-7 exposed rat lungs (Sager et al. 2022) may be considered an adaptive response due to the increased demand for the AMs to facilitate phagocytosis and thus detoxification of the toxic MWCNT-7 particles. This view is further supported by the significant and MWCNT-7 dose-dependent enrichment in the functional and canonical pathway categories viz. recruitment of phagocytes, activation of macrophages, and phagosome formation as well as differential expressions of the genes involved in those categories (Sager et al. 2022). The interaction between the toxic particles deposited in the lungs and the AMs may result in the activation of the macrophages and the ensuing release of various signaling molecules. Activation of AMs by inhaled MWCNT-7 was supported by the dose-dependent increase in the transcript, *RETNLA1*, a marker for macrophage activation (Nair et al. 2009), in the MWCNT-7 exposed rat lungs (Sager et al. 2022). Cytokines/chemokines are prominent among the signaling molecules released by the activated AMs (Becker et al. 1991). The transcripts for several cytokines/chemokines viz. *CCl2*, *CCl7*, *CCl9*, *CCL22*, *CXCl2*, *CXCl3*, *CXCl6*, and *CXCL10* were significantly overexpressed in MWCNT-7-exposed rat lungs. The cytokines/chemokines released by the macrophages are involved in the recruitment of additional macrophages to the lungs, activation of the macrophages, and phagocytosis of toxic particles by the AMs and, therefore, are important determinants in deciding the fate of the particles with respect to their potential to induce lung toxicity.

The involvement of inflammation in lung toxicity and the potential for adverse health effects resulting from the exposure to MWCNT is well established (Dong and Ma 2016a; Porter et al. 2010; Poulsen et al. 2016). Results of the bioinformatic analysis of the gene expression data, in addition to supporting the induction of inflammation in MWCNT-7-exposed rat lungs, provided insights into the mechanisms underlying the induction of inflammation. Recruitment of phagocytes, primarily PMNs, from blood to the lungs is one of the first responses to pulmonary exposure to MWCNTs. The interaction of the MWCNT-7 particles deposited in the lungs with the AMs and/or the lung epithelium results in the release of pro-inflammatory signaling molecules. The recruitment of PMNs from the circulatory system to the lungs is a complex, multistep process that involves their tethering, activation, adhesion to the endothelium, rolling, and transmembrane release (Mizgerd 2002). The entire process is controlled by signaling molecules, including cytokines/chemokines that belong to the CC and CXC families (Mizgerd 2002). The overexpression of the

transcripts for pro-inflammatory cytokines/chemokines in the lungs and pulmonary infiltration of the PMNs exhibited a response to the cumulative MWCNT-7 dose to which the rats were exposed, suggesting a possible relationship among overexpression of cytokines/chemokines, pulmonary infiltration of PMNs, and the lung injury detected in MWCNT-7 exposed rats (Sager et al. 2022).

Results of the bioinformatic analysis of the SDEGs, in addition to supporting the involvement of inflammation in MWCNT-7-induced lung toxicity, provided information regarding the potential molecular mechanisms underlying the toxicity. This included the enrichment of several inflammation-related IPA biological functions such as inflammatory response and inflammation of airways and canonical pathways, such as acute phase response signaling, LXR/RXR activation, complement system, and TREM1 signaling as well as the significant increases in the expression levels of several genes belonging to those categories in MWCNT-7-exposed rat lungs (Sager et al. 2022). The acute phase response is a rapid inflammatory response to defend tissues/organs against invading microorganisms (Perez 2019) and injury resulting from exposure to toxic agents (Saber et al. 2014). However, enhanced activation of the acute phase response and excessive release and accumulation of acute phase response signaling molecules may result in tissue damage and toxicity. Serum amyloid is a marker for acute phase response (Sack 2018) and a significant overexpression of the *Serum amyloid A like 1 (Saal1)* transcript was detected in MWCNT-7-exposed rat lungs. Similarly, the transcript for another gene, *Orosomucoid 1 (ORM1)*, involved in the acute phase response and inflammation (Alfadda et al. 2012; Ligresti et al. 2012) was highly overexpressed in MWCNT-7-exposed rat lungs, which further supported the involvement of acute phase response in the lung inflammation and toxicity induced by MWCNT-7, similar to that resulting from exposure to other particles that are toxic to the lungs (Joseph et al. 2021; Sager et al. 2020). Like the acute phase response, activation of the complement system is another important mechanism involved in the immune and inflammatory responses to tissue injury by toxic particles (Pandya and Wilkes 2014). Complement proteins, regulators of the complement system, are synthesized by lung epithelial cells (Strunk et al. 1988; Varsano et al. 2000) and fibroblasts (Volanakis 1995) in response to the release of pro-inflammatory cytokines (Huber-Lang et al. 2002). The complement proteins, in turn, may function as chemoattractants facilitating the infiltration of PMNs into the lungs to result in the induction of inflammation, such as that was detected in MWCNT-7 exposed rat lungs (Sager et al. 2022). The transcripts for many of the genes involved in complement response, viz. *Complement C1s (C1s)*, *Complement 3 (C3)*, *Complement 6 (C6)*, *Complement factor B (CFB)*, *Complement C1q A chain (C1QA)*, *Complement C1q B chain (C1QB)*, *Complement C1qC chain (C1QC)*, *Integrin subunit alpha M (ITGAM)*, *Integrin subunit alpha X (ITGAX)*, and *Integrin subunit beta 8 (ITGβ8)* were overexpressed in rat lungs and their overexpression was dependent on the cumulative MWCNT-7 dose to which the rats were exposed. Taken together, these findings suggested the activation of the complement system and its potential involvement in the MWCNT7-induced lung inflammation and injury detected in the rats. Triggering receptor expressed on myeloid cells 1 (TREM1) is a receptor that belongs to the immunoglobulin family of cell surface receptors. The association between activated TREM1 and its partners, the toll-like receptors (TLR), facilitates the release of cytokines, such as MCP-1, MIP-2, and TNF-α (Bleharski et al. 2003; Ornatowska et al. 2007), resulting in a pro-inflammatory response. The significant overexpression of the transcripts for *TREM1* and several *TLRs* as well as the elevated BALF levels of cytokines (MCP-1, MIP-2, and TNF-α) detected in the lungs are suggestive of the potential involvement of the TREM1 signaling pathway in the induction of lung inflammation and injury that resulted from inhalation exposure of rats to MWCNT-7.

Generation of reactive oxidants and the resulting oxidative stress, similar to the induction of inflammation, is another major mechanism involved in the toxicity and pathology associated with

the exposure to MWCNT (Srivastava et al. 2011). The gene expression data and findings of the bioinformatic analysis supported the MWCNT-7-induced oxidative stress in rat lungs (Sager et al. 2022). Nrf2 is a redox-sensitive transcription factor that regulates the expression of key genes involved in protecting the biological system through the detoxification of toxic reactive oxidants (Kensler et al. 2007). MWCNT-7-induced oxidative stress, pulmonary inflammation, and fibrosis were significantly higher in the Nrf2 knock-out mouse, compared with the wild-type mouse (Dong and Ma 2016a), suggesting a protective role for Nrf2 in the deleterious effects associated with MWCNT-7 exposure. Genes that are members of the canonical pathway, Nrf2-mediated oxidative stress, were significantly overexpressed in the lungs of MWCNT-7-exposed rats. For example, *Superoxide dismutase 2 (SOD2)* – an Nrf2-regulated gene that was significantly overexpressed in the lungs of the MWCNT-7-exposed rats is primarily responsible for the generation of hydrogen peroxide, a toxic ROS, by dismutation of the superoxide anion. The *NADPH oxidase organizer 1 (NOXO1)* gene involved in the generation of toxic superoxide anion (Katsuyama et al. 2012) was also highly overexpressed in MWCNT-7-exposed rat lungs. Other genes involved in oxidative stress response such as *Lactoperoxidase (LPO)* (Sharma et al. 2013), *Hemeoxygenase 1 (HMOX1)* (Nakashima et al. 2018), and *Lipocalin 2 (LCN2)* (Roudkenar et al. 2007), also exhibited a significant and dose-dependent overexpression in MWCNT-7-exposed rat lungs. The absence of efficient detoxification of the ROS generated may facilitate their accumulation in lungs, resulting in oxidative stress and injury, including excessive inflammation and fibrosis, as previously reported in the case of the Nrf2 knock-out mouse, in response to MWCNT-7 exposure (Dong and Ma 2016b).

Pulmonary exposure of rats and mice to MWCNT is known to cause lung fibrosis (Porter et al. 2010; Dong et al. 2015; Kasai et al. 2015, 2016). Bioinformatic analysis of the gene expression data obtained from the lungs of MWCNT-7-exposed rats also provided information relevant to the molecular mechanisms involved in fibrosis. The *Serpine family E member 1 (SERPINE1)* gene plays a pivotal role in lung diseases through its involvement in chronic inflammation (Tiwari et al. 2016), tissue remodeling (Chen et al. 2021), and fibrosis (Ghosh and Vaughan 2012). Furthermore, *SERPINE1* gene polymorphism is a risk factor for respiratory diseases (Chen et al. 2021) and inhibitors of SERPINE1 protein have therapeutic application in treating upper respiratory diseases (Huang et al. 2012). Considering the role of the *SERPINE1* gene in respiratory diseases, the significant overexpression of its transcript detected in the lungs of rats should be of functional significance to MWCNT-7-induced lung fibrosis. Other genes whose expressions were significantly higher in MWCNT-7-exposed rat lungs, compared with the controls, and potentially involved in MWCNT-7-induced fibrosis were *secreted phosphoprotein 1 (SPP1)*, *TIMP metallopeptidase inhibitor 1 (TIMP1)*, *chitinase, acidic (CHIA)*, *adenosine A1 receptor (ADORA1)*, *pentraxin 3 (PTX3)*, and *matrix metallopeptidase 12 (MMP12)*. Osteopontin, the protein product of the SPP1 gene, through the activation of the TGF-β signaling pathway, facilitates the activation and differentiation of fibroblasts to myofibroblasts – a critical step involved in fibrosis (Dong and Ma 2017a). A similar role for TIMP1 in MWCNT-7-induced lung fibrosis through the activation and proliferation of fibroblasts has been identified (Dong and Ma 2017b). Extracellular matrix (ECM) formation, a critical event in fibrosis, is a complex process that involves the synthesis and degradation of ECM proteins, most notably collagen. The significant overexpression of *ADORA1*, *CHIA*, *MMP12*, and *PTX3* in MWCNT-7 exposed rat lungs, their established role in the maintenance of the ECM (Churg and Wright 2005; Cronstein 2011; Lee et al. 2012; Pilling et al. 2015), and the increase in collagen detected in the lungs should be considered as evidence for their potential involvement in the MWCNT-7-induced lung fibrosis in rats.

The IARC, based on the results of animal studies, has classified one form of the MWCNT, MWCNT-7, the same material used in our rat lung toxicity study, as a Group 2B human carcinogen

(IARC (International Agency for Research on Cancer) 2017). Whereas MWCNT-7 was identified as a tumor promoter in a mouse model (Sargent et al. 2014), prolonged inhalation exposure to MWCNT-7 alone resulted in significant increases in pre-neoplastic and neoplastic lesions in rat lungs, suggesting that MWCNT-7 is a complete carcinogen (Kasai et al. 2016). Despite the identification of MWCNT-7 as a carcinogen, the mechanisms underlying its carcinogenicity were not well understood. The absence of positive results in the Ames test (Ema et al. 2012) suggested that the carcinogenicity of MWCNT-7 may not be due to its role as a direct mutagen. On the other hand, changes in chromosome number were detected in cultured cells in response to exposure to MWCNT-7 (Asakura et al. 2010; Siegrist et al. 2014), attributing the induction of aneuploidy as a potential mechanism involved in the MWCNT-7-induced carcinogenesis. In fact, in immortalized human airway epithelial BEAS-2B cells, the chromosomal aberrations induced by MWCNT-7 exposure were attributed to the disruption of the centrosomes and interference with the mitotic spindle apparatus in association with cell cycle block (Siegrist et al. 2014). Furthermore, the genetically damaged, aneuploid BEAS-2B cells exhibited increased colony formation, a feature characteristic of the transformed phenotype, further supporting the induction of aneuploidy as a potential mechanism underlying the neoplastic potential of MWCNT-7.

The gene expression data obtained in our rat inhalation exposure and toxicity study (Sager et al. 2022) further supported the previously reported role of MWCNT-7 as a carcinogen (Kasai et al. 2016) and suggested potential mechanisms underlying its carcinogenicity. The biological function category, lung cancer, was significantly enriched in response to MWCNT-7 exposure in rat lungs (Sager et al. 2022) similar to that reported by Guo et al. (2012) in mice exposed to MWCNT-7 by pharyngeal aspiration. Similarly, cell cycle control of chromosomal replication and kinetochore metaphase signaling pathway were two of the significantly enriched canonical pathway categories in MWCNT-7 exposed rat lungs (Sager et al. 2022). The enrichment of these IPA categories and the overexpression of the genes involved in those categories, similar to the results of the lung toxicity assessment parameters employed in the study, were dependent on the MWCNT-7 dose to which the rats were exposed, suggesting the potential involvement of the overexpressed genes in mediating MWCNT-7-induced lung toxicity/carcinogenesis.

Faithful replication of chromosomal DNA is essential for the stable propagation of genetic information during cell division. This requires that chromosomal DNA replication, a very complex process, regulated by the expression of multiple genes, takes place only once per cell division. The genes that regulate the chromosomal DNA replication during cell division/cycle belong to three major classes – origin recognition complex (ORC), minichromosome maintenance (MCM), and cell division cycle (CDC), each regulating distinct and critical events involved in the faithful replication of chromosomal DNA (Dabral et al. 2019; Hizume et al. 2013; Perl et al. 2019). Overexpression of the ORC, MCM, and CDC genes resulting in excessive production of the encoded proteins and modulation of their functions may result in abnormal chromosomal DNA replication, leading to cell transformation and cancer (Issac et al. 2019; Semple and Duncker 2004; Tomita et al. 2011). Members of the ORC, MCM, and CDC gene families were significantly overexpressed in the lungs of MWCNT-7-exposed rats suggesting their potential involvement in lung cancer due to exposure to MWCNT-7 (Sager et al. 2022).

Another major event in cell division, relevant to carcinogenesis, is the replication of chromosomes taking place during metaphase and their subsequent segregation, equally, during anaphase to result in daughter cells with the same number of chromosomes as that of the parental cell. The kinetochore-spindle assembly formation plays a critical role in the equal segregation of the sister chromatids to the daughter cells. The entire process is controlled by proteins that belong to the KMN network consisting of Knl1, Mis12, and Ndc80 complexes (Varma and Salmon 2012).

Transcripts for the genes whose protein products play important roles in the kinetochore-spindle assembly formation, through their participation in the KMN network, were found significantly overexpressed in MWCNT-7-exposed rat lungs (Sager et al. 2022). The established role of KMN genes in oncogenesis (Bai et al. 2019; Gao et al. 2022), the observation that MWCNT-7-induced aneuploidy resulted in the acquisition of an oncogenic phenotype in human airway epithelial cells (Siegrist et al. 2014), and significant overexpression of the KMN network gene transcripts in MWCNT-7-exposed rat lungs may suggest that deregulation of expression of the genes involved in kinetochore metaphase signaling pathway is a potential mechanism underlying MWCNT-7-induced cancer.

Similar to the studies conducted in our laboratory, several other investigators have also employed gene expression profiling to determine the mechanisms underlying MWCNT-induced lung injury as well as to identify pulmonary disease outcomes of exposure to ENMs. Concordant changes in DNA methylation and lung global gene expression profiles were detected in mice at 56 days following administration of a single dose of MWCNT-7 by pharyngeal aspiration (Scala et al. 2021). Functional analysis of the gene expression data obtained from mice identified pathways involved in muscle contraction, immune system/inflammation, and extracellular matrix formation as the most affected ones and their potential involvement in MWCNT-induced lung toxicity. Poulsen et al. (2015) demonstrated that pulmonary transcriptional responses indicative of lung fibrosis varied for carbon nanotubes with differences in physical and chemical properties. In a mouse toxicogenomics study (Labib et al. 2016), an adverse outcome pathway framework approach was employed to identify key biological events linking MWCNT exposure to lung fibrosis. The investigators administered MWCNT with different physical and chemical properties in mouse lungs and major pathways perturbed by the exposures were identified by global gene expression profiling and bioinformatic analysis of the resulting data. The results obtained from the study showed that similar biological pathways were perturbed by different MWCNT particles in spite of the differences in their physical and chemical properties. Based on these results, the authors concluded that transcriptomic data can be employed as an effective mechanism-based approach to derive acceptable levels of exposure to nanomaterials in situations where epidemiological data are not available.

Toxicogenomics of MWCNT – Human Studies

Compared with the large number of animal studies, there have been only fewer human studies that investigated the potential toxicity and the health effects associated with exposure to MWCNT. In a study reported by Shvedova et al. (2016), the changes in the peripheral blood transcriptome of workers, in response to their exposure to MWCNT in a manufacturing facility, were investigated. The study population consisted of workers ($n = 8$) who had direct contact with an aerosol containing MWCNT for at least six months. A population consisting of professionals and/or technical staff ($n = 7$) from the same manufacturing facility with no known history of exposure to MWCNT served as the control. The detection of MWCNT particles in the PBZ of the workers engaged in manufacturing MWCNT confirmed their occupational exposure to the ENM. Global expression profiles of the long noncoding RNA (ncRNA), mRNA, and microRNA (miRNA) were determined in the peripheral blood samples collected from the control and MWCNT-exposed workers by microarray (lncRNA and mRNA) or NGS (miRNA) analysis. The resulting data were analyzed to determine the differences in the expression profiles of the RNA molecules in the blood samples obtained from the control and the MWCNT-exposed subjects. Significant differential expression

(fold change ≥/± 1.5 and $p < 0.05$) of 977 ncRNAs, 785 mRNAs, and 11 miRNAs were detected between the blood samples obtained from the MWCNT-exposed and the nonexposed workers. The authors further reported that the changes in expressions of the ncRNAs and mRNAs were prominent in three workers who had higher MWCNT exposure compared with the remaining five workers with relatively lower levels of MWCNT exposure. Bioinformatic analysis of the expression data was performed to determine the potential disease outcomes associated with MWCNT exposure as well as the molecular mechanisms potentially involved in the diseases. Many of the dysregulated mRNAs and miRNAs detected in the blood samples obtained from the MWCNT-exposed workers were mostly associated with pulmonary inflammation and fibrosis, in agreement with the results obtained from several rodent toxicity studies (Ellinger-Ziegelbauer and Pauluhn 2009; Labib et al. 2016; Dong et al. 2016; Sager et al. 2022). The findings of the bioinformatic analysis of the transcriptomics data also suggested the potential for MWCNT exposure to result in cardiovascular and carcinogenic outcomes.

The finding that the blood transcriptome responds to pulmonary exposure to MWCNT in mice (Snyder-Talkington et al. 2015) and humans (Shvedova et al. 2016) may have significant application in the prevention of adverse health effects potentially resulting from exposure to MWCNT. It is known that gene expression changes taking place in the blood, in response to exposure to toxic agents, are relevant to target organ toxicity (Bushel et al. 2007; Sellamuthu et al. 2011a, 2012). Furthermore, the superior sensitivity of gene expression changes as indicators of toxicity compared with biochemical and histological changes taking place in the target organs has been established (Umbright et al. 2010). Blood gene expression signatures have been employed as highly sensitive surrogate biomarkers for the detection of early toxicity resulting from exposure to acetaminophen (Bushel et al. 2007) and crystalline silica (Sellamuthu et al. 2011a). Since small quantities of the blood required to conduct transcriptome profiling can be obtained by a minimally invasive procedure, blood gene expression signatures can be developed and employed to monitor human exposure to MWCNT. Additionally, understanding the molecular mechanisms involved in the toxicity, through bioinformatic analysis of the transcriptomics data, may provide the opportunity to develop therapeutics targeting the genes and/or the encoded proteins. Therefore, transcriptomic studies have the potential to prevent the development of life-threatening health effects that are likely associated with exposure to MWCNT.

Disclaimer:

The findings and conclusions in this report are those of the author and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention.

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