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NEW PERSPECTIVES FOR IN VITRO RISK ASSESSMENT OF MULTIWALLED CARBON NANOTUBES: APPLICATION OF COCULTURE AND BIOINFORMATICS

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Nanotechnology is a rapidly expanding field with wide application for industrial and medical use; therefore, understanding the toxicity of engineered nanomaterials is critical for their commercialization. While short-term in vivo studies have been performed to understand the toxicity profile of various nanomaterials, there is a current effort to shift toxicological testing from in vivo observational models to predictive and high-throughput in vitro models. However, conventional monoculture results of nanoparticle exposure are often disparate and not predictive of in vivo toxic effects. A coculture system of multiple cell types allows for cross-talk between cells and better mimics the in vivo environment. This review proposes that advanced coculture models, combined with integrated analysis of genome-wide in vivo and in vitro toxicogenomic data, may lead to development of predictive multigene expression-based models to better determine toxicity profiles of nanomaterials and consequent potential human health risk due to exposure to these compounds.

The interaction with and manipulation of nanomaterials have been evident since the fourth century A.D. and have become one of the most heavily invested technologies in the past decade (http://nano.gov). Defined by the U.S. National Nanotechnology Initiative as "the understanding and control of matter at the nanoscale, at dimensions between 1 and 100 nanometers, where unique phenomena enable novel applications," nanotechnology is the study of materials that have at least one dimension in the range of 1–100 nm (http://nano.gov). Nanomaterial may refer to materials occurring naturally through gas-to-particle conversions, combustion reactions, microbial activity, and mineral weathering, or to engineered materials that are intentionally produced to exploit their novel properties for use in a variety of commercial fields (Nel et al., 2006; Oberdorster et al., 2005b; Wigginton et al., 2007). From appliances, automotive, and electronic goods to kitchenware, food additives, and personal health items, the list of goods incorporating nanotechnology is ever growing (Scholars, 2011).

The rapid expansion of nanotechnology requires the assessment of the interaction of nanoparticles with biological systems. A framework for the responsible development of nanotechnology has been suggested to fully evaluate nanomaterial, determine the potential risks, and implement appropriate safety measures (www.nanoriskframework.com). Occupational exposures during synthesis, incorporation into products, and product use and disposal, as well as public exposures through environmental contamination and the use of commercial goods, are concerns

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for the growing industry (Oberdorster, 2010). Although numerous short-term studies have been performed to determine a product's safety before market, the effects of chronic exposure are largely unknown (Teow et al., 2011). Nanotoxicology, the study of the interaction of nanoparticles with biological systems, aims to determine the resulting responses to nanoparticle exposure and time frame in which these responses occur (Castranova, 2011). Nanoparticle uptake occurs through multiple routes of administration, such as inhalation or dermal contact, during industrial processes, as well as dermal and gastrointestinal tract absorption through medical devices, food products, and environmental contamination (Hagens et al., 2007). The method of absorption of nanomaterials may affect their toxicity due to differences in penetration and clearance. Once exposure occurs, the diverse characteristics of nanoparticles, their various physical, chemical, and catalytic properties, induce a range of responses within a biological system. Translocation from the site of absorption and interaction with proteins may alter the metabolism, distribution, and elimination or possible accumulation of the nanomaterial in the body (Zhao and Castranova, 2011). In addition, the metabolism of nanomaterial may contribute to unexpected toxicity due to altered chemical composition and structural characteristics. One critical characteristic of nanoparticles is their high surface area per mass as compared to larger particles of the same composition. As particle size decreases, the relative surface area increases, enhancing the possibility for electronic disruption, structural defects, and the number of potential surface oxidant reactive sites, thus intensifying the possible biological reactivity of the material (Kim et al., 2010; Nel et al., 2006; Pacurari et al., 2010). Multiple in vitro studies showed that, on an equal mass basis, nanosized particles produced an increased cytotoxic effect compared to their fine-sized counterparts, and this effect was attributable to differences in surface area (Ding et al., 2009; Sager et al., 2008; Warheit et al., 2009a). Additional attributes of engineered nanoparticles, such as surface coatings and treatments or the ability to agglomerate, may also alter their toxic potential (Nel et al., 2006).

The unique physical and reactive properties of nanomaterials can lead to unpredictable adverse effects; therefore, understanding the pharmacokinetics of such materials is paramount to accurately assess toxicity. To determine the ultimate effect of nanoparticles after exposure, it is important to know the distribution to target organs, accumulation and persistence in these organs, and the adverse effects exerted by the nanoparticle on individual cells so that safe exposure levels can be determined (Hagens et al., 2007). In vivo studies of nanoparticle exposure by various routes (intravenous, intraperitoneal, topical, subcutaneous, and pulmonary [inhalation, intratracheal instillation, or pharyngeal aspiration]) in animal studies showed a range of distribution patterns dependent on the route of administration with possible translocation of nanomaterial to the blood, liver, spleen, lung, brain, heart, and kidneys. Nanoparticle exposure most often results in the initiation of an inflammatory response and may exhibit the ability to induce fibrosis in both lung and liver after inhalation and intravenous administration, respectively (Hagens et al., 2007; Li et al., 2010).

While the necessity of animal studies to accurately determine toxicity profiles of nanomaterials is unquestionable, there is a current effort to shift toxicological testing from an in vivo observational standpoint to an in vitro predictive model using advanced in vitro techniques to mimic the in vivo situation. Building on the tenets sent forth by W. M. S. Russell and R. L. Burch in 1959, the need to refine experiments so as to minimize animal distress, reduce the number of animal experiments performed while maintaining quality information, and eventually replace a majority of animal testing with in vitro methods is a focus of current U.S. Environmental Protection Agency (EPA) and National Toxicology Program (NTP) strategic plans to shift in vivo animal studies to in vitro assays and computational modeling for toxicity assessment in humans (Collins et al., 2008; Maynard et al., 2006; Russell and Burch,

1959). In addition to obvious cost-saving strategies, in vitro biochemical and cell-based assays will allow for high-throughput screening that, when combined with computational toxicology, can infer predictive outcomes upon human exposure while refining and prioritizing animal studies (Collins et al., 2008).

Although monoculture systems of cells are the predominant model system for in vitro toxicological testing, many studies found little correlation between results obtained in vitro and those obtained in vivo (Sayes et al., 2007; Seagrave et al., 2003; Warheit et al., 2009b). Seagrave et al. (2003) determined, through their analysis of the toxicity of gasoline and diesel emissions, that particles that exerted the least cytotoxic effect in vitro had the most potent effect in vivo. Conversely, the most potent particle in vitro was among the least potent during in vivo studies. This same conflict was seen by both Sayes et al. (2007) and Warheit et al. (2009b). Sayes et al. (2007) found little to no correlation in cytotoxic and inflammatory markers between in vivo exposures to five different particle types when compared to in vitro results. This conclusion was echoed by Warheit et al. (2009b), as cytokine profiles of lung epithelial cells and macrophages in monoculture exposed to both fine and nanoscale zinc oxide (ZnO) did not correlate with potency profiles determined after instillation or inhalation in vivo. Therefore, there is a need for improved cell culture techniques and models for high-throughput screening to predict nanomaterial toxicity before in vivo testing. A more advanced cell culture system in which multiple cell types are grown together to allow cross-talk between cells needs to better mimic the in vivo situation and provide more reliable information of nanoparticle toxicity when compared to conventional monoculture systems.

Newly developed high-throughput-based global gene expression profiling techniques have made it possible to identify key predictive gene signatures from various specimens. In addition, it has become increasingly important to use gene expression signatures identified with bioinformatics methods for toxicity prediction, risk assessment, and screening (Afshari et al., 2011; Shi et al., 2006). In 2005, the U.S. EPA established the National Center for Computational Toxicology (NCCT) to integrate high-throughput screening into its testing program. The combined computational modeling of in vivo and in vitro toxicogenomics data could prioritize critical toxicity pathways so as to design intervention strategies and identify novel biomarkers for risk assessment of human health ramifications (Collins et al., 2008). Recently, bioinformatic analyses of genomic data were identified by both the U.S. Food and Drug Administration (FDA) and the U.S. EPA as key opportunities to advance personalized medicine and environmental risk assessment (Dix et al., 2006; Frueh, 2006; Lesko and Woodcock, 2004). The MicroArray Quality Control (MACQ) project shows inter- and intraplatform reproducibility of gene expression measurements, substantiating the feasibility of establishing a framework for the use of microarrays in clinical and regulatory settings for drug discovery, medical diagnosis, and risk assessment (Shi et al., 2006).

While there are numerous naturally occurring and engineered nanoparticles, each with their own distinct physicochemical characteristics, this review (1) focuses on multiwalled carbon nanotubes (MWCNT) as an example of one type of nanoparticle, (2) examines their effects on both in vivo and in vitro systems, (3) discusses the need for a combination of both advanced in vitro systems and in vivo evaluation to fully comprehend the toxicity profile of such nanomaterials, and (4) provides new perspectives for the use of computational systems biology in human health assessment of MWCNT exposure. The use of in vitro coculture studies coupled with genome-wide computational toxicogenomic analysis is a novel approach to the assessment of nanotoxicology that provides a high-throughput and cost-effective strategy to the refinement of in vivo toxicological testing. While this review focuses on the use of advanced in vitro techniques for MWCNT risk assessment, several comprehensive and insightful reviews are available for an in depth analysis of MWCNT and other nanomaterials (Johnston et al., 2010; Pacurari et al., 2010; Zhao and Castranova, 2011).

MWCNT IN VIVO STUDIES

First described by Iijima in 1991, MWCNT are structures that resemble sheets of graphite rolled into hollow tubes forming concentric cylinders inside one another. The number of concentric cylinders varies, ranging from 2 up to 50 or more, with the diameter of the tube staying within the nanometer (nm) range even though the length of the cylinder may reach into the micrometer (μm) range (Iijima, 1991). MWCNT possess many unique physicochemical properties. Their closed cage phenotype and hexagonal pattern of carbon rings permit an inert environment and electron sharing, which establishes aromaticity and efficient electronic conductivity. The strong carbon bonds permit immense strength, which enables MWCNT to undergo extreme strain without breakage or deformity (Yakobson and Smalley, 1997). Of particular interest to the biomedical industry, the tube-like channels within MWCNT exert strong capillary forces resulting in the ability of MWCNT to act as nanocarriers for a variety of drug delivery systems (Ajayan, 1999).

The unique and valuable physicochemical properties of MWCNT put them at the forefront of the booming nanotechnology field; therefore, the need to understand their toxicity profile after both short-term and chronic exposure is necessary. Respiratory studies to determine the pulmonary effects of MWCNT lead the toxicology field in evaluation, since aerosolization during production, use, and disposal is a main route of occupational MWCNT exposure. Muller et al. (2005) determined that, after intratracheal instillation, MWCNT had the ability to produce both inflammatory and fibrotic responses in the rat lung. An increase in lactate dehydrogenase (LDH) activity as well as tumor necrosis factor-α (TNF-α) was indicative of inflammation up to 15 d after particle exposure. Sixty days after exposure, increased collagen levels and granuloma formation were observed, indicating that MWCNT had the ability to produce persistent injury and also induce a fibrotic response after a single exposure (Muller et al., 2005). Additional in vivo studies of MWCNT exposure confirmed an elevation in inflammatory and fibrotic activity in mouse lung, uptake of the MWCNT into alveolar macrophages (AM) and other alveolar cells, and impaired lymphatic drainage (Mercer et al., 2010, 2011; Porter et al., 2010). Injection of MWCNT into the peritoneal cavity or scrotum so as to mimic exposure to the chest mesothelial lining was found to elicit a length-dependent inflammatory response and granuloma formation in both mice and rats, followed by the slow development of mesothelioma (Muller et al., 2009; Poland et al., 2008; Sakamoto et al., 2009; Takagi et al., 2008).

The ability of the body to clear nanomaterial after either intentional or unintentional exposure affects the toxicity profile of the material. Upon inhalation, nanoparticles travel through the upper respiratory tract and deposit into the lungs. The nanoscale diameter of MWCNT contributes to their pulmonary deposition as the majority of MWCNT may be able to pass through the conducting airways and reach the alveolar regions. Indeed, Mercer et al. (2010) reported that after aspiration of MWCNT in a mouse model, 18% of the particles deposit in the conducting airways while 81% deposit in the alveolar region of the lung. Analysis of the distribution pattern after inhalation yielded similar results (D. Porter, 2012). While MWCNT that deposit on the conducting airway are rapidly cleared, those in the alveolar region may pass through the alveolar epithelial lining by either a paracellular or transcellular route and reach the lung interstitium, where time-dependent fibrosis occurs (Mercer et al., 2010, 2011). MWCNT further translocate to the extracellular space and lymphatics and may be transported from subpleural tissue to the intrapleural space (Mercer et al., 2010). The ability of macrophages to engulf and destroy foreign material is essential to nanoparticle clearance and biopersistence, and this clearance is dependent upon the size and shape of the particle. Nanosized particles were found to induce numerous toxicological responses in comparison to their fine-sized counterparts, including a greater inflammatory response in the lung and longer

retention time in the body, which may increase the time of exposure to nanomaterials even after initial exposure has passed (Oberdorster, 1994, 2010). The fibrous nature of MWCNT results in a high aspect ratio as the length of MWCNT greatly surpasses its nanoscale diameter. Toxicity of high-aspect-ratio nanomaterials has been well studied in the case of asbestos. Rigid asbestos fibers have the increased ability to induce pulmonary inflammation, fibrosis, mesothelioma, and cancer after exposure as compared to more flexible forms of asbestos due to their long, inflexible form (Mossman and Churg, 1998; Mossman et al., 2011; Osinubi et al., 2000). This pulmonary toxicity is thought to progress through the inability of macrophages to efficiently engulf and remove long fibrous materials. As fiber length increases, macrophages cannot fully surround the invading particle and become "frustrated," secreting proinflammatory cytokines and eliciting an immune response (Donaldson et al., 2010). The rigid fibrous shape of MWCNT is analogous to rigid fibrous asbestos; therefore, a similar mode of frustrated phagocytosis was postulated to occur after MWCNT exposure (Donaldson et al., 2010; Pacurari et al., 2010). Comparative analysis of pulmonary exposure studies with MWCNT, flexibile asbestos, and rigid asbestos conclude that all have the ability to induce inflammatory and fibrotic responses to some degree, due in part to a high aspect ratio and biopersistence (Donaldson et al., 2010; Mercer et al., 2010; Muller et al., 2005). The rate of clearance of MWCNT, or lack thereof, and the role of clearance rate in the etiology of MWCNT-induced inflammatory and fibrotic responses require further study (Donaldson et al., 2010).

MWCNT MONOCULTURE IN VITRO STUDIES

In vitro testing determined that MWCNT display a range of adverse effects. Reactive oxygen species (ROS) production is a common characteristic of MWCNT exposure in various cell types (He et al., 2011; Pacurari et al., 2012; Ye et al., 2009). ROS are primarily created by mitochondria as a by-product of aerobic respiration and as an antimicrobial agent by phagocytic cells. Direct mitochondrial damage from MWCNT exposure results in depolarization of the mitochondrial membrane potential, leading to increased ROS production in lung fibroblasts (He et al., 2011). The oxidative stress that arises from the generation of these ROS exerts a number of deleterious effects on the cell, including activation of proinflammatory signaling cascades, DNA damage, and cell death (Nel et al., 2006). Using an in vitro model of lung epithelial cells, Ye et al. (2009) determined that ROS production increased after alveolar epithelial (A549) cell exposure to MWCNT. This rise in ROS resulted in enhanced production of the proinflammatory mediator interleukin (IL)-8 due to ROS-induced activation of the redox-sensitive transcription factor nuclear factor (NF)-κB. Additional studies in A549 cells also found that MWCNT exposure elevated the expression levels of IL-1β, IL-6, IL-10, and macrophage chemoattractant protein (MCP) 1, and this was also attributable to an increase in ROS-induced NF-κB signaling (He et al., 2011). Therefore, MWCNT possess the ability to induce the production of multiple inflammatory markers, most likely through ROS production and induction of NF-κB signaling. In addition to upregulation of inflammatory mediators, MWCNT were able to induce a concentration-dependent activation of genes associated with oxidative stress and apoptosis, such as p53 and caspase-3, at both the mRNA and protein level in A549 cells (Srivastava et al., 2011). The aspect ratio of MWCNT was determined to be a key component in MWCNT-induced cytotoxicity of human embryonic lung cells, and this effect was independent of trace metal contamination (Kim et al., 2010; Porter et al., 2010). Exposure of keratinocytes to MWCNT in vitro induced both IL-8 and IL-1β production, also implicating MWCNT in potential inflammatory reactions after dermal exposure and potentially contributing to inflammation and progressive disease states of the skin (Witzmann and Monteiro-Riviere, 2006). With regard to MWCNT-induced toxicity to the

intestine due to occupational and environmental ingestion, no observable cytotoxicity was induced by either pristine or oxidized MWCNT and uptake of MWCNT into Caco-2 human intestinal enterocytes was not observed (Clark et al., 2012). Although MWCNT did interact with the microvilli of the cells, their effect was minor, and the adverse effect of MWCNT in the intestine requires further study (Clark et al., 2012).

Witzmann and Monteiro-Riviere (2006) determined overall MWCNT-induced in vitro proteomic alterations in keratinocytes through two-dimensional (2D) gel electrophoresis and peptide mass fingerprinting of significantly altered proteins at 24 and 48 h postexposure. These significantly altered proteins were involved in diverse cellular signaling pathways such as apoptosis, metabolism, and growth and differentiation (Witzmann and Monteiro-Riviere, 2006). A proteomics approach to determine overall significant protein alterations to a monoblastic leukemia cell line after MWCNT exposure was also undertaken by Haniu et al. (2010, 2011). Alterations in proteins associated with the cell cycle, transcription, and metabolism, among others, were noted after MWCNT exposure (Haniu et al., 2010). While these toxicoproteomic-based evaluations suggest a novel approach to the determination of MWCNT-induced toxicity, their use in in vitro cellular systems involving relevant exposure concentrations in lung and in vivo models remains to be determined.

In vitro exposure to MWCNT also induced double-strand DNA breaks and chromosomal aberrations (CA) in rat lung epithelial cells (Muller et al., 2008). It was proposed that the mode of action was either a direct interaction of the MWCNT with DNA molecules or secondary actions such as ROS production. The ability of MWCNT to induce CA may be due to the structure of MWCNT themselves and their ability to interact with and replace various components of the cellular machinery. MWCNT possess the ability to interact with alpha- and beta-tubulin subunits in a cell-free system and self-assemble into functional tubulin–MWCNT hybrids with the MWCNT acting as an internal structure onto which the alpha- and beta-tubulin subunits adsorb (Dinu et al., 2009). While this unique trait of MWCNT may be beneficial from a bioengineering standpoint, it raises concern that a similar mechanism may occur in vivo after either intentional or unintentional MWCNT exposure. Microtubules are dynamic structures of alpha and beta subunits that continually polymerize and depolymerize in response to signals from the cell, while MWCNT are fibrous, strong, static structures that do not deform easily in size or shape. If MWCNT had the ability to enter into a cell and incorporate into microtubules during assembly, they might also have the ability to interact with the mitotic spindle and thus interfere with chromosome segregation. It is here that MWCNT may play a detrimental role in chromosomal segregation, resulting in aberrant chromosome numbers and creating a potential disease state that is characterized by loss of chromosomal stability (Sargent et al., 2010). In addition, DNA strand breaks are a consequence of an altered balance of ROS production. As an increase in ROS production is a well-known characteristic of MWCNT exposure, ROS-induced DNA damage occurs, additional ROS are produced, and a cycle is created through which chronic diseases may progress (Ma, 2010). Ultimately, MWCNT exposure may lead to apoptosis of exposed cells, as MWCNT were reported to induce cell death (He et al., 2011; Kim et al., 2010; Srivastava et al., 2011).

In vivo, MWCNT produced a rapid and persistent interstitial fibrosis, which was associated with translocation of MWCNT into the alveolar walls (Mercer et al., 2011; Porter et al., 2010). In vitro studies showed that dispersed MWCNT induced the secretion of fibrogenic mediators from lung epithelial cells and collagen production by lung fibroblasts (Mishra et al., 2011). The production of transforming growth factor (TGF) β and platelet-derived growth factor (PDGF) from macrophages upon MWCNT exposure is also indicative of a profibrogenic environment (He et al., 2011).

While the physical attributes of MWCNT alone are enough to produce a harmful effect after exposure, their chemical composition is also a vital piece of information to determine

toxicity. Multiple techniques are available for the production of MWCNT, such as electric-arc discharge and laser vaporization, which generate MWCNT through the high-temperature vaporization of a graphite target in an inert atmosphere followed by chemical vapor deposition. Metal catalyst particles are added into the reaction to lower the temperature needed for efficient deposition, and Awasthi et al. (2005) reported that through these production techniques, metal contaminants may become entrapped in the nanomaterials. Iron, nickel, and cobalt are commonly used metal catalysts that may become incorporated into MWCNT and potentially elicit an adverse effect of their own. Pulskamp et al. (2007) suggested that purification techniques, which lower the amount of metal contamination in MWCNT samples, may alleviate some of the cellular toxicities seen upon MWCNT exposure, thus suggesting that the toxicity of MWCNT is due to metal impurities and not the MWCNT themselves. However, studies showed that even highly purified MWCNT may still induce cytotoxicity and that MWCNT in acellular systems do not have the ability to form ROS, indicating that their potential toxicity is independent of metal contaminants (Porter et al., 2010; Tsukahara and Haniu, 2011). A summary of the in vitro MWCNT monoculture studies presented is provided in Table 1.

MWCNT: IN VITRO VERSUS IN VIVO RESULTS

For in vitro assays to be effective and predictive screening tests of nanoparticle in vivo bioactivity, the following factors need to be considered. In vitro MWCNT concentrations need to be relevant to in vivo lung burdens, and effort needs to be made to disperse MWCNT for in vitro testing so that structure sizes are similar to inhaled MWCNT structures upon aerosolization of dry material. In vitro assays need to be selected that are most relevant to in vivo mechanisms of action. In general, the physicochemical properties of MWCNT and other nanoparticles need to be well characterized and compared to appropriate controls for efficient toxicity testing (Warheit and Donner, 2010).

A review of the current literature indicates that in vitro studies of MWCNT pulmonary toxicity use exposure concentrations as high as 200 μg/ml (Table 1). Such in vitro doses are much higher than achieved in pulmonary exposure studies in animal models. Relevant in vitro concentrations are achieved by using MWCNT mass concentrations/surface area of cultured cells that mimic MWCNT mass burdens/alveolar epithelial surface area from animal studies. Porter et al. (2010) determined, based upon studies of alveolar surface area and peak airborne concentrations of MWCNT in laboratory and occupational settings, relevant concentrations of MWCNT for use during in vivo exposure (Han et al., 2008; Maynard et al., 2004; Stone et al., 1992). Taking into consideration peak MWCNT aerosol levels found previously in an occupational setting, MWCNT mass median aerodynamic diameter, minute ventilation, and human alveolar epithelium surface area, Porter et al. (2010) concluded that a 10-μg MWCNT exposure to a mouse would approximate 1 mo of exposure to a human in a work environment with aerosol concentrations of 400 $\mu g/m^3$ MWCNT. In occupational settings where airborne levels of MWCNT were found to be lower, the 10-μg dose of MWCNT was suggested to approximate exposure levels between 9 mo and 7.5 yr (Maynard et al., 2004; Porter et al., 2010). Porter et al. (2010) noted that aerosol levels of MWCNT at 400 $\mu g/m^3$ suggest human exposure to be 226 μg MWCNT/m^2 of human alveolar epithelium per month of exposure. In vivo experiments demonstrated that a 10-μg exposure to MWCNT induced significant inflammation that returned to control levels 7 d after exposure. Additional exposures at 20, 40, or 80 μg MWCNT induced persistent inflammation up to 56 d postexposure, with signs of fibrosis at the 80-μg dose (Mercer et al., 2011; Porter et al., 2010). These concentrations can be extrapolated for use during in vitro studies with 226 μg MWCNT/m^2 corresponding to a MWCNT concentration of less than 1 μg/ml

TABLE 1. Summary of MWCNT Monoculture In Vitro Studies

Reference	Model System	Concentration	Time periods	Conclusions
Clark et al. (2012)	Caco-2 (epithelial), RAW264.7 (macrophage)	50 μg/ml	24 h	Caco-2 cells show neither cytotoxicity nor uptake of MWCNT after 24-h exposure. Interaction of MWCNT with microvilli produced minor abnormalities.
Hanui et al. (2010)	U937 (monoblastic leukemia)	100 μg/ml	96 h	As-grown and thermally treated MWCNT induced proteomic alterations at 96 h.
He et al. (2011)	BEAS-2B (epithelial), WI38-VA13 (fibroblast), A549 (epithelial), RAW264.7 (macrophage)	0, 1, 2, 5, 10, 20, and 200 μg/ml	0, 0.5, 1, and 2 h (macrophage) 16, 24, and 96 h (epithelial/fibroblast)	MWCNT induce dose-dependent increases in cell death/inhibition of proliferation at 96 h. Exposure of cells to 20 μg/ml for 16 h induced mitochondrial damage, ROS production, NF-κB activation, and IL-1β, IL-6, TNFα, IL-10, and MCP-1 production. Exposure of cells to 20 μg/ml for 24 h increased mRNA and protein levels of TGFβ1 and PDGF.
Kim et al. (2010)	WI-38 (fibroblast)	0, 12.5, 25, 50, 100, and 200 μg/ml	24, 48, and 72 h	MWCNT of both low and high aspect ratio induce inhibition of cell proliferation at all concentrations and time points and increase LDH activity at 25–200 μg/ml for 24 h and 50–200 μg/ml for 48 and 72 h.
Mishra et al. (2011)	BEAS-2B (epithelial), A549 (epithelial), CRL1490 (fibroblast)	0, 0.02, and 0.6 μg/cm^2		MWCNT decrease cell viability in a dose-dependent manner, induce fibrogenic mediators from epithelial cells and fibroblasts, and induce collagen production from fibroblasts.
Muller et al. (2008)	RLE (epithelial), MCF-7 (epithelial)	0, 10, 25, 50, 100, and 150 μg/ml	6, 24, and 48 h	In a dose-dependent manner, MWCNT induced LDH release (24 h), apoptosis (6 h), and micronucleus formation (24 or 48 h). MWCNT induce clastogenic and aneugenic events.
Pacurari et al. (2012)	HMVEC (endothelial)	0, 2.5 μg/ml	0.5, 1, 4, 8, 24, and 50 h	HMVEC engulf MWCNT and induce gap formation, ROS production, and actin filament remodeling beginning at 1 h, MWCNT induce monolayer permeability consistently over 50 h. MWCNT induce migration (24 h) MCP-1 (24 h), and ICAM-1 (8 h) production.
Pulskamp et al. (2007)	NR8383 (macrophage), A549 (epithelial)	0, 5, 10, 50, and 100 μg/ml	24, 48, and 72 h	MWCNT induced a dose-dependent decrease in cell viability using an MTT assay but not WST assay (24 h) or PI staining (24, 28, 72 h), an increase in ROS production (24 h), and a loss of mitochondrial functionality (24 h). MWCNT did not induce inflammatory meditators.
Srivastava et al. (2011)	A549 (epithelial)	0, 0.5, 1, 5, 10, 50, and 100 μg/ml	3, 6, 12, 24, 48, and 72 h	MWCNT were internalized in a dose-dependent manner. 50 and 100 μg/ml MWCNT induced a decrease in cell viability (24 and 48 h) while 10 and 50 μg/ml induced significant levels of ROS (6, 12, and 24 h). MWCNT induce a dose and time dependent effect on GSH reduction (6, 12, 24 h), LPO production (24 h), and catalase activity (24 h). Apoptotic bodies were significant at 10 and 50 mg/ml (72 h) and apoptotic genes were altered in a dose-dependent manner (12 h). Protein levels were altered at 10 and 50 μg/ml (24 h)
Tsukahara and Haniu (2011)	BEAS-2B (epithelial)	0.1, 1, 5, 10, 30, and 100 μg/ml	12, 24, and 72 h	MWCNT internalized into cells (12 h) and, in a dose-dependent manner, decreased cell metabolism and viability (24 h) and increased protein levels of IL-6 and IL-8 (24 h)
Witzmann and Menteiro-Riviere (2006)	HEK (keratinocyte)	0, 400 μg/ml	24 and 48 h	MWCNT induced significant changes in protein levels of IL-8 (24 and 48 h) and IL-1β (48 h); 36 total proteins had altered expression at 24 h while 106 proteins had altered expression at 48 h.
Ye et al. (2009)	A549, BEAS-2A (epithelial)	25, 50, 100, 150, and 200 μg/ml	2, 24 and 72 h	MWCNT induce a concentration-dependent effect on cell viability at 72h, IL-8 protein and mRNA expression from 25- 150 mg/ml at 24 h, ROS production from 50-150 mg/ml at 24 h, and NF-kB activity from 50-150 mg/ml at 2 h.

in standard tissue culture protocols. Higher in vivo exposure levels (20, 40, or 80 μg) correlate to in vitro concentrations approximately less than or equal to 1 μg/ml. Therefore, low concentrations of MWCNT in in vitro studies are suggested to be the most biologically relevant.

In addition to biologically relevant doses, it is also necessary to understand the behavior of different nanoparticles in cell culture media with regard to particle settling, diffusion, and aggregation as the size, density, and physicochemical properties of a particular nanomaterial may affect its interaction with neighboring nanoparticles, cell culture media, and underlying cells when placed into a liquid suspension (Oberdorster et al., 2005a; Teeguarden et al., 2007). In vivo studies concerning the fibrotic response to aspiration of a CNT suspension indicate that dispersed CNT are more fibrogenic than agglomerated CNT (Mercer et al., 2008). Shvedova et al. (2008) showed that the generation of a dry CNT aerosol for inhalation exposure produces structure sizes significantly smaller than CNT suspensions in phosphate-buffered saline used for aspiration exposure. As a consequence, CNT inhalation resulted in a greater pulmonary response than aspiration of poorly dispersed CNT. Therefore, to mimic response to inhaled MWCNT, properly dispersed MWCNT need to be used for in vitro test systems. Indeed, dispersed CNT exhibit fibrogenic activity with fibroblast cell cultures, as expected from animal studies, while agglomerated preparations do not (Wang et al., 2010a, 2010b). To alleviate the various modifications that suspension of MWCNT in cell culture media may induce, in vitro models may be modified with the addition of an air–liquid interface to mimic exposure in vivo. Epithelial cells grown on permeable supports are hydrated basolaterally while their apical surface is exposed to a controlled air and exposure environment, inducing a well-differentiated epithelial culture that mimics the epithelial state in vivo (Brandenberger et al., 2010; de Bruijne et al., 2009; Fulcher et al., 2005; Hill and Button, 2012; Lenz et al., 2009). Evaluations of such systems with nanomaterials suggested direct deposition of particles with the alleviation of agglomeration, diffusion, and particle loss associated with submerged culture (Brandenberger et al., 2010). While the effects of MWCNT in an in vitro air–liquid interface environment remain to be resolved, this in vitro method is a promising alternative to fully submerged cell culture experiments with regard to nanoparticle-induced pulmonary toxicity.

Nel et al. (2006) proposed that oxidative stress may be a predictive paradigm for the bioactivity of nanoparticles. Indeed, Rushton et al. (2010) found that for a set of spherical particles, in vitro generation of ROS by alveolar macrophages was predictive of their inflammatory potential in the lung. In contrast, the fibrogenic potential of CNT in vivo does not appear to require persistent oxidative injury and inflammation (Porter et al., 2010; Shvedova et al., 2005). At doses relevant to lung burdens in animal studies, CNT do not induce substantial oxidant injury. However, low concentrations of CNT in vitro stimulate proliferation and collagen production in cultured lung fibroblasts and induce TGFβ production by lung epithelial cells (Mishra et al., 2011; Wang et al., 2010a, 2010b). Therefore, for in vitro assays to be predictive of in vivo responses, they need to be directed toward mechanisms of action relevant to in vivo response.

Currently, there are some disparate conclusions concerning the toxicity of MWCNT when comparing monoculture in vitro results to those seen in vivo that suggest the need for enhanced cell culture techniques. An in vitro study of the circulatory compatibility of MWCNT determined that MWCNT in vitro had the ability to induce the intrinsic pathway of coagulation, as well as increase platelet numbers. Functionalization of the MWCNT by either amidation or carboxylation enhanced this procoagulant activity. As seen with previous studies of nanomaterial, in vivo results were not concordant and showed an opposite effect with diminished coagulant activity and reduced platelet numbers upon functionalization (Burke et al., 2011). Functionalization of MWCNT with carboxylic polyacid groups facilitated internalization into a monoculture of macrophages in vitro and showed oxidative and inflammatory

responses similar to those of nonfunctionalized MWCNT; however, in vivo results suggested that the oxidative and inflammatory responses to functionalized MWCNT were increased when compared to nonfunctionalized MWCNT (Tabet et al., 2011). A study of the ability of MWCNT to irritate both the skin and eyes determined that results were concordant between in vitro and in vivo results with regard to skin, in that both showed little to no irritation; however, in vitro results indicated that MWCNT were nonirritating to the eye, while in vivo results suggested irritation (Kishore et al., 2009). Recent studies showed that MWCNT are able to induce genotoxic effects in both in vivo and in vitro assays; however, the extent to which these effects occur in vivo compared to in vitro is still a matter of discussion (Ema et al., 2012; Kato et al., 2012).

COCULTURE MODELS TO PREDICT NANOPARTICLE TOXICITY

One method to improve the discrepancies between cellular and animal studies is the use of coculture cell models. These complex models mimic the cellular cross-talk of an in vivo signaling environment and are an expanding in vitro methodology to better predict the potential in vivo effects of nanomaterials. While animal models may be the most relevant to human occupational or environmental exposures, in vitro testing allows for more effective evaluation of cellular functions and molecular pathways. In addition, in vitro assays are low in cost and afford the high rate of throughput required for screening the growing number of nanomaterials being developed for commercialization (Collins et al., 2008; Rothen-Rutishauser et al., 2008). As inhalation of nanomaterials remains the predominant potential route of human exposure, coculture models aiming to mimic the alveolar-capillary units of the lung are essential for the understanding of the complex interactions of cells in the lung. Although air–liquid interface using epithelial cells alone has been the focus of numerous studies to allow for biologically relevant exposure methods of the lung epithelium, the additional need for microvascular or immune cells in the culture system is essential to allow for cross-talk between pulmonary cell types and incorporation of air–liquid interface into coculture models (Brandenberger et al., 2010; Diabate et al., 2008).

To simulate the alveolar-capillary unit in vitro, various cell types of epithelial, endothelial, and immune origin are grown on opposite sides of a permeable filter so that physiologically relevant barriers to nanomaterial in vivo can be created. Exposure of epithelial cells in the apical well of the two-part chamber, whether by submerged culture or air–liquid interface, aims to mimic the exposure to nanomaterial after inhalation, while cells representing the interstitium or vasculature in the basolateral chamber receive signals from the exposed epithelial barrier and respond accordingly. This is an advantage over conventional cell culture techniques, as monoculture does not have the ability to demonstrate interplay between different cell types (Hermanns et al., 2004). In a case of silica exposure, a coculture model of apically seeded human lung H441 cells and basolaterally seeded human microvascular endothelial ISO-HAS-1 cells showed less toxicity, as evidenced by impaired barrier function, upon exposure to silica nanoparticles when compared to separate monocultures (Kasper et al., 2011). This coculture also determined that low levels of silica exposure produced increased ICAM-1, IL-6, and IL-8 expression, suggesting an inflammatory response, where monocultures exerted little or no reaction. As no nanoparticles were found in the basolateral chamber of the coculture after apical exposure and endothelial cells alone in the basolateral chamber were not able to induce the same effect, Kasper et al (2011) suggested that this difference in cellular signaling was due to cross-talk between the two cells types in coculture. Through a similar evaluation of the interaction between H441 epithelial cells and human microvascular endothelial cells, the nanocarrier polyethyleneimine (PEI) was determined to have differential cellular uptake between monoculture and coculture systems

during an in vitro test of gene delivery. While PEI was readily detectable in both epithelial and endothelial cells during monoculture, coculture of cells showed little to no detectable uptake of PEI after epithelial exposure under the same conditions (Hermanns et al., 2010). These coculture results echo similar findings obtained in vivo. While preclinical studies of adenoviral gene delivery determined that small doses of vector might amend defective chloride transport in a monoculture in vitro model of cystic fibrosis, aerosolized delivery of the vector to both mouse and human lung disease models resulted in low efficiency of uptake and little improvement in chloride transport (Grubb et al., 1994). Hermanns et al. (2010) proposed that cells behave differently in coculture, that an epithelial/endothelial coculture representation of the alveolar-capillary barrier behaves in a more in-vivo-like manner than either cell type alone, and that paracrine signaling between cell types may be responsible for the establishment of a tighter epithelial barrier in coculture than that found in monoculture. The effect of cell–cell communication in coculture may represent a more physiologically relevant model for the study of nanoparticle uptake, toxicity, and potential cellular mechanisms, as well as providing a better model for lung drug delivery.

In addition to modeling lung epithelial and endothelial barrier function, a model of the interaction between lung epithelial cells and the surrounding immune cells is also necessary for an in vitro prediction of a possible immune response to nanoparticle exposure. Coculture models of lung epithelial and endothelial barriers that incorporate various cells of the immune system found that cellular reactions to nanomaterials were either mitigated or amplified when compared to monoculture (Muller et al., 2010). Coculture of lung epithelial cells with macrophages suggested greater sensitivity to nanomaterial as a significant rise in inflammatory markers was induced compared to separate monoculture (Wottrich et al., 2004). A coculture of lung epithelial and endothelial cells that also incorporated macrophages and mast cells into the epithelial chamber resulted in greatly increased inflammatory signals above those predicted by monoculture after particulate matter exposure. This coculture response more closely matched inflammatory effects seen in vivo (Alfaro-Moreno et al., 2008). Interestingly, cocultures of lung epithelial cells with mast cells and macrophages gave a different cytokine profile than a similar coculture that also included an endothelial layer, suggesting that even amongst cocultures, the interplay between multiple cell types is evident, increasing the validity of coculture systems over conventional monoculture (Alfaro-Moreno et al., 2008). While the effects of nanoparticle exposure on coculture of pulmonary cells are the most prevalent, coculture models have also been adopted to determine the effect of nanomaterial exposure on other organ systems, such as brain and intestinal tract, with results similar and congruent to in vivo studies (Bouwmeester et al., 2011; Meng et al., 2007).

Although there are currently no published coculture studies of MWCNT, the lack of association between results obtained in vitro and results obtained in vivo suggests that more relevant concentrations, nanoparticle dispersion, mechanisms, and methods of exposure must be incorporated into in vitro techniques to study MWCNT toxicity. In addition, improved in vitro techniques could be combined with in silico computational analysis of both in vivo and in vitro data so as to mimic and predict in vivo effects based upon genomic data modeling for risk assessment.

COMPUTATIONAL TOXICOGENOMICS TO INFER TOXICITY PATHWAYS AND MOLECULAR MECHANISMS

Genome-wide expression analysis could infer critical toxicity pathways and aid molecular mechanistic studies for intervention of environmental diseases by evaluating the relevance of signaling pathways representing significantly perturbed genes (Afshari et al., 2011; Hamadeh et al., 2010). A few studies used in vivo or in vitro genome-wide mRNA expression profiling to infer toxicity pathways induced by MWCNT in a rat model (Alazzam et al.,

2010; Ellinger-Ziegelbauer and Pauluhn, 2009; Peng et al., 2010). A combination of benchmark dose (BMD) methods, microarray data, and Gene Ontology (GO) functional annotations was applied to estimate a dose range of adverse biological processes in toxicity tests (Thomas et al., 2007, 2011, 2012b). Using the BMD methods, the expression profile of each gene was fitted with parametric models, including a linear model, a second-degree polynomial model, a third-degree polynomial model, and a power model; the best-fit model was selected to derive the safe dose range of this gene (Thomas et al., 2007, 2011, 2012b). The median BMD of all genes belonging to a biological process was used to present the safe dose range of this process. Alternatively, the dose-response models could also be fitted with parametric models including exponential, linear, Gaussian, quadratic, and sigmoid models (Burgoon and Zacharewski, 2008; Kopec et al., 2010). In these studies, histological phenomena were observed but were not used in gene expression modeling to identify gene activities or biological processes that resemble the observed histological patterns (Burgoon and Zacharewski, 2008; Kopec et al., 2010; Thomas et al., 2007, 2011, 2012b). Further, these studies did not delve into rigorous simulation of pathway activities associated with the observed in vivo time-course and dose-response histopathological phenomena. In an effort to mathematically model gene transcriptional activities resembling the observed in vivo histological patterns, clustering approaches and Bayesian decomposition methods were explored in previous studies. Traditional clustering approaches partitioned each gene into a single coexpression group, although genes were often coexpressed in different groups depending on time or dose conditions (Tamayo et al., 1999; Waring et al., 2001; Yeung and Ruzzo, 2001). More sophisticated methods based on Bayesian decomposition were computationally inefficient and thus not practically scalable to a genome-wide model to infer pathway activities (Moloshok et al., 2002; Ochs et al., 2009).

In order to overcome the limitations of these methods, recent studies developed a novel and computationally efficient model based on nonnegative matrix factorization, Monte Carlo Markov chain simulation, and gene set enrichment analysis (Devarajan, 2008; Dymacek and Guo, 2011; Lee and Seung, 1999; Russell and Norvig, 2003; Subramanian et al., 2005). This method is nonparametric and is thus robust to model pathway activities resembling any time-course dose-response histopathological patterns observed in animal studies. This novel computational model identified relevant processes of MWCNT-induced inflammation and fibrosis from dose-response time-series DNA microarray data in mouse lungs following MWCNT aspiration (Dymacek and Guo, 2011). For the identified processes, their transcriptional activities closely resembled the observed time-course dose-response histopathological patterns of lung inflammation and fibrosis in animal studies. Predicted mRNA gene expression changes from the analysis of in vivo microarray data were validated in vitro through mRNA and protein expression analysis of small airway epithelial cells exposed to MWCNT (Snyder-Talkington et al., personal communication, 2012). The in vitro mRNA and protein expression levels of selected genes matched those seen after in vivo exposure and thus indicated that the computational model was sufficient to predict cellular changes after in vivo exposure that may be validated through in vitro mechanistic studies (Snyder-Talkington et al., personal communication, 2012). The evaluation of the most significantly represented pathways throughout this comprehensive evaluation might inform further mechanistic studies focusing on these signaling pathways for investigation of MWCNT-induced inflammation and fibrosis, thus refining the mechanistic studies from an observational standpoint to one of predictive outcomes.

Comparative toxicogenomic analyses of gene expression data from in vivo animal studies and in vitro human cells identified critical biological processes and toxicity pathways (Deng et al., 2010; Doktorova et al., 2012; Heise et al., 2012; Kienhuis et al., 2009; Ord et al., 2005; Robinson et al., 2011). In these studies, genes showing concordant expression changes in both in vivo and in

vitro systems were selected and their functional pathway involvements evaluated with bioinformatics tools such as Gene Ontology and Ingenuity Pathway Analysis. Using these comparative in vivo and in vitro analyses, multiple genes are selected to construct a model to predict the in vivo and in vitro toxicity response (Cheng et al., 2011). Proteomic techniques, including two-dimensional gel electrophoresis (2-DE) and liquid chromatography tandem mass spectrometry (LC-MS/MS), were also used in the search for toxicity mechanisms and biomarkers in both in vivo and in vitro studies (Van Summeren et al., 2012).

In order to facilitate the screening and prioritization of chemicals for in vivo testing, the U.S. EPA initiated the ToxCast project and the Tox21 Consortium to characterize in vitro biological activities of chemicals, including toxicogenomic data. A similar initiative, REACH, was implemented in Europe to test both new and existing chemicals. By using chemical structure descriptors, in vitro assays, and genomic data, a mathematic model could be constructed to classify in vivo responses of each chemical (Thomas et al., 2009, 2012a). Potentially, this classifier could infer the in vivo toxicity response of a new chemical. Results showed that the predictive power of the in vitro assays was not significantly different from the chemical structure metrics and that aggregating in vitro assays with selected gene markers reduced predictive performance (Thomas et al., 2012a). These results indicate the challenges of using in vitro assays in combination with toxicogenomic analysis to predict the in vivo response during chemical screening. More sophisticated gene selection methods beyond simple *t*-tests may be required to accomplish such tasks.

COMPUTATIONAL GENOMIC DATA MODELING TO IDENTIFY BIOMARKERS FOR RISK ASSESSMENT IN HUMANS

Integrated in vivo and in vitro assays combined with genome-wide analyses might reveal toxicity pathways for mechanistic studies and identify biomarkers for risk assessment. Nevertheless, as discussed earlier, there is often a gap between in vivo and in vitro MWCNT-induced toxicity studies and risk assessment in humans. Computational analysis of microarray data may prioritize critical toxicity pathways and identify innovative biomarkers for risk assessment in exposed individuals (Collins et al., 2008). In vivo and in vitro gene expression signatures associated with specific histopathological alteration phenotypes might be identified from toxicogenomics data to predict human health ramifications and risk assessment based on similarities of gene expression profiles between the exposed in vivo and in vitro samples and those of humans (Amin et al., 2004; Bushel et al., 2007; Hamadeh et al., 2002, 2004; Luhe et al., 2003; Paules, 2003; Powell et al., 2006).

Studies suggested that animal model-based gene expression profiling may successfully predict target organ toxicities for numerous human diseases (Aubrecht and Caba, 2005; Bushel et al., 2007; Newton et al., 2004; Nuwaysir et al., 1999). Specifically, in the study by Bushel et al. (2007), blood gene expression signatures identified from acetaminophen (APAP)-exposed rats could separate APAP-intoxicated patients from unexposed controls, indicating that gene expression data from peripheral blood cells can provide valuable information about environmental disease well before liver damage is detectable by classical parameters. The unique advantage of such studies is the ability to detect toxic injury at the molecular level and thus identify molecular events that lead to organ injury long before the clinical symptoms occur.

In a recent genome-wide expression analysis of mouse lungs exposed to MWCNT, gene signatures were identified to predict lung cancer risk and progression in human patient samples (Guo et al., 2012). This study estimated the health hazards of MWCNT exposures by comparing the similarity between MWCNT-induced gene alterations and gene expression profiles in patient samples. Lung adenocarcinoma patients having gene expression patterns more similar to those in the MWCNT-treated mice were found to have more aggressive tumors with a

poor clinical outcome, whereas patient tumors showing a gene expression pattern less similar to that in the MWCNT-treated mice demonstrated less metastatic potential with a relatively better clinical outcome (Guo et al., 2012). These MWCNT-induced gene signatures were also shown to be associated with increased risk for developing lung cancer using animal and human patient data (Guo et al., 2012). The microarray results were confirmed in quantitative real-time polymerase chain reaction (qRT-PCR) analysis of a set of previously identified lung cancer biomarkers and related signaling pathway genes (Guo et al., 2008; Pacurari et al., 2011; Wan et al., 2010). This study used the nearest centroid classification method to predict human lung cancer progression by measuring the correlation between mouse gene expression data and patient gene expression profiles. This algorithm is robust to account for different microarray platforms and, in this case, different species, and was successfully used to classify breast cancer subtypes in clinics based on gene expression profiles quantified with different microarray platforms (Perou et al., 1999, 2000; Sorlie et al., 2003; van 't Veer et al., 2002). This approach could estimate the risk in individual patients based on gene expression profiles, which is different from the risk assessment methods such as BMD or dose-response models that estimate a safe dose range for a gene or a biological process (Burgoon and Zacharewski, 2008; Kopec et al., 2010; Thomas et al., 2007, 2011, 2012b).

In the study by Guo et al. (2012), lung tissue specimens were collected from mice exposed to MWCNT from 1 to 56 d postexposure, which was not sufficient time for mice to develop lung cancer. However, in vivo animal model-generated gene profiling revealed information that approximated the complexity of the human body and its cellular, biochemical, and molecular systems that are involved in responses to chemical agents. The unique advantage of this study was the ability to detect responses at the molecular level that may lead to pathology long before clinical symptoms are detectable. The emphasis of this study was on the prediction of potential risk or toxicity at an early stage. The ability of MWCNT-induced gene sets to correlate with carcinogenesis of lung cancer patients provided justification for a further long-term study to determine the temporal association between MWCNT-induced gene alterations and development of precancerous lesions and/or tumors in the mouse lung, which is currently underway at the National Institute for Occupational Safety and Health (Qian, personal communication, 2012). Given the nature of biomarker research, clinically applied biomarkers need to be validated in the following three phases: retrospective studies, prospective evaluation, and clinical trials. The study by Guo et al. (2012) that utilized multiple retrospective patient cohorts to validate the identified biomarker genes is an initial step, that is, the identification of potentially useful gene signatures for (1) further study, (2) development of a surveillance approach for early detection of lung cancer, and (3) prognosis with MWCNT in the workplace.

Gene expression profiling has yielded two commercially available, clinically used breast cancer prognostic tests, MammaPrint and Oncotype DX (Paik et al., 2004; van 't Veer et al., 2002; van de Vijver et al., 2002). In these routine clinical gene tests, mRNA expression, not protein expression, is used to predict clinical outcome in patients. The commonly accepted approach in these biomarker studies is to use mRNA expression for clinical diagnosis or prognosis, rather than protein expression, as mRNA quantification is considered reliable for clinical tests, whereas current protein expression assays, such as immunohistochemistry or Western blots, are semiquantitative and thus not favored for developing multigene assays as clinical tests.

CONCLUSIONS

The growing nanotechnology field calls for the improved, rapid, and cost-effective testing of nanoparticle toxicology. Hazards of nanoparticle exposure exist during synthesis and disposal, commercial use, and various nanomedicine diagnostic and therapeutic interventions (Oberdorster, 2010; Zhao and

Castranova, 2011). Although multiple studies attempted to collate the adverse health effects of nanoparticles for efficient risk assessment, current knowledge remains insufficient for the development of an informed conclusion (Becker et al., 2011; Card et al., 2011; Dhawan and Sharma, 2010; Fadeel and Garcia-Bennett, 2010; Kuhlbusch et al., 2011; Oberdorster, 2010). As regulatory agencies and advocacy groups call for a shift from primary observational science through animal testing to a more predictive science through the development of sophisticated in vitro approaches, nanotoxicology methods needs to adapt to the changing era (Collins et al., 2008; Stokstad, 2009). Although at present in vitro assays cannot fully replicate the intricate balance of interactions in vivo, there is a distinct difference in signaling between cells in monoculture and cells in coculture (Hermanns et al., 2010; Kasper et al., 2011; Muller et al., 2010; Wottrich et al., 2004). This change in cellular signaling is attributed to the cross-talk between the different cell types in culture. Although validation of coculture in vitro studies to mimic the in vivo environment remains difficult, the ability to incorporate cellular cross-talk into in vitro models allows for more relevant cellular signaling. Preliminary data suggest that in a coculture model of human small airway epithelial cells (SAEC) and human microvascular endothelial cells (HMVEC), HMVEC display increased ROS production, enhanced actin disruption, and decreased VE-cadherin expression at the cell membrane after epithelial exposure to 1.2 μg/ml MWCNT. An overall rise in VEGFA protein expression in both chambers, in addition to an increase in HMVEC angiogenic ability, suggests cross-talk between the two cells types after epithelial exposure (Snyder-Talkington, personal communication). In addition, genome-wide mRNA microarray analysis suggests multiple gene expression changes when comparing SAEC and HMVEC grown in monoculture to SAEC and HMVEC grown together in coculture, even in the absence of MWCNT exposure (Snyder-Talkington, personal communication, 2012). Therefore, cells of pulmonary origin may possess distinct cellular signaling variations based upon their growth conditions and studies are underway to determine the correlation of mRNA expression among monoculture, coculture, and in vivo gene expression (Snyder-Talkington, personal communication). The use of low concentrations of adequately dispersed MWCNT and appropriate mechanistic endpoints demonstrate the biological relevance of this system and illustrate the potential of the coculture model to study MWCNT-induced pulmonary toxicity.

While some studies on the influence of various nanomaterials in coculture systems were conducted, the effects of MWCNT in coculture systems remain to be evaluated. As the number of commercial applications involving MWCNT advances, so must in vitro testing technology to accurately predict the effects of purity, length, width, and functionalization on toxicity. The use of MWCNT in coculture models coupled with in silico gene expression profiling is a necessary development to adequately determine their toxicity in a manner most relevant to in vivo exposure studies. Coculture models will also advance the ability to elucidate the underlying cellular mechanisms governing bioactivity and development of relationships between physicochemical properties and bioactivity to assist assignment of an untested nanoparticle into a relative toxicity category, that is, high, mid, or low toxicity. The toxicity category, along with process parameter information such as degree and duration of exposure, may be used for control banding, a qualitative strategy for determining the degree of exposure controls necessary in the absence of official exposure limits.

The institution of in vitro assay systems that parallel results from in vivo models will provide a means for the determination of cellular mechanisms associated with nanotoxicity. These enhanced in vitro data, coupled with in silico bioinformatics-based gene expression profiling, might help to determine the underlying mechanisms associated with biological responses to nanomaterial exposure and inform refinement of in vivo toxicological testing and risk assessment (Figure 1). Traditional toxicological studies focus on observations at

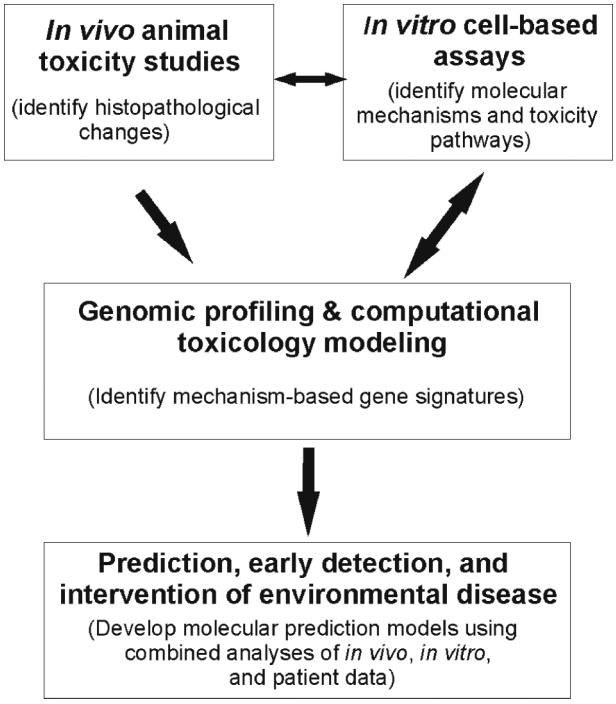

FIGURE 1. A schematic of the proposed strategy to incorporate in vivo and in vitro toxicological data with computational toxicological modeling for prediction and early detection of disease and relevant mechanisms.

the level of disease-specific models in vivo, whereas this review proposes a strategy to focus on broad mechanism-based biological observations in vitro with the support of in vivo studies. With this approach, data derived from in vitro systems are applied together with those from in vivo animal studies, as well as from human origin, in order to identify novel biomarkers, early characteristic genomic patterns, and mechanisms of MWCNT-induced pulmonary toxicity in humans. This strategy will lead to the development of methods for early detection and interventions of MWCNT-induced pulmonary diseases, particularly fibrosis, in humans.

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