

Characterization of 2D and 3D Models of Particle-Induced Fibrogenesis

by

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B.S., Virginia Polytechnic Institute and State University, 2013

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This dissertation by Alysha E. Simmons is accepted in its present form by the Pathobiology Graduate Program as satisfying the dissertation requirements for the degree of Doctor of Philosophy

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As an undergraduate student at Virginia Polytechnic Institute and State University, Alysha E. Simmons received two Bachelor of Science degrees in Biological Sciences and Animal and Poultry Sciences in 2013. In the fall of 2014, she started graduate work in the Pathobiology Program at Brown University and received the NIEHS NRSA T32 Pre-Doctoral Fellowship from 2015 to 2019. Alysha joined the labs of Dr. Ian Wong and Dr. Agnes Kane in the School of Engineering and Department of Pathology, respectively, to pursue her dissertation research titled, “Characterization of 2D and 3D Models of Particle-Induced Fibrogenesis.”

Journal Publications

Bordeleau, F., Wang, W., **Simmons, A.**, Antonyak, M. A., Cerione, R. A., Reinhart-King, C. A. (2020). “Tissue transglutaminase 2 regulates tumor cell tensional homeostasis by increasing contractility.” *Journal of Cell Science*. 133(1).

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Coutermarsh-Ott, S., **Simmons, A.**, Capria, V., LeRoith, R., Wilson, J., Heid, B., Phillipson, C., Qin, Q., Hontecillas-Magarzo, R., Bassaganya-Riera, J., P-Y Ting, J., Dervisis, N., Allen, I. (2016). “NLRX1 suppresses tumorigenesis and attenuates histiocytic sarcoma through the negative regulation of NF-B signaling.” *The Journal of Immunology*. 194(7), pp.3369-3380.

Williams, T.M., Leeth, R.A., Rothschild, D.E., Coutermarsh-Ott, S.L., McDaniel, D.K., **Simmons, A.E.** Heid, B., Cecere, T.E. and Allen, I.C. (2015) “The NLRP1 inflammasome attenuates colitis and colitis-associated tumorigenesis.” *The Journal of Immunology*. 194(7), pp.3369-3380.

Williams, T., Leeth, R., Rothschild, D., McDaniel, D., Coutermarsh-Ott, S., **Simmons, A.**, Kable, K., Heid, B., Allen, I. (2015). “Caspase-11 attenuates gastrointestinal inflammation and experimental colitis pathogenesis.” *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 308(2), G139-G150.

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0.1 Dedication

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Chapter 1

Introduction

1.0.1 Potentially Biopersistent, Fibrogenic Contaminants in the Environment and Workplace

Nanomaterials are defined as any material with at least one dimension below 100 nanometers, with a variety of chemical compositions and shapes that enable exceptional physiochemical properties. Nanomaterials are becoming widely utilized for a variety of consumer products, but may also be produced as a byproduct of manufacturing. As a result, nanoparticles can become airborne in industrial facilities as nano-sized or ultrafine aerosolized particulates through routine industrial processing or cleaning of machinery. Carbon nanoparticles can be accidentally released into the environment through poor industrial by-product disposal or vehicle combustion processes, i.e., vehicle exhaust. Environmental contamination can also occur from the laundering of textiles with incorporated nanoparticles, combustion apart from vehicles (campfires, cooking fires, trash fires), leakage from products at trash disposal sites, or during the aerosolized application of coatings or paints. Carbon nanoparticles embedded in nanoelectronics also exhibit degradation associated with long term exposure to heat and electricity, resulting in changes to the properties of the parent nanoparticle, in addition to any changes to nanoparticle bioavailability as a result of

interaction with the environment. These nanoparticle transformations require several research approaches to identify the wide range of potential toxicities associated with exposure. Ideally, assays that could measure multiple types of endpoints in a high-throughput, cost-effective, and reproducible manner after acute exposures would offer the most value to the scientific and industrial communities.

Without personal protective equipment, industrial workers can be exposed to high concentrations of aerosolized nanoparticles for extended periods of time. Nanoparticle deposition in the human lung could predispose industrial workers to several pulmonary diseases in addition to pulmonary fibrosis [1-4]. Translocation of particulates through the pleura into the interstitial space or the vasculature can result in their systemic circulation of nanoparticles and increased risk to other organ systems. Increases in morbidity from cardiovascular disease and fibrosis from the accumulation of nanoparticles in the liver, kidneys, and spleen have been observed [5-8]. To aid in the protection of industrial workers, the Environmental Protection Agency (EPA) and Occupation Safety and Health Administration (OSHA) recognize several particle toxicants derived from industrial processes and aid in the implementation of regulations to protect workers. The two most common routes of exposure to aerosolized particulates in the natural environment and the workplace are through ingestion and inhalation. The objectives and conditions of the screening tools evaluated for this thesis research are informed exclusively by the context of inhalation exposures as toxicity data attributed to ingestion exposures is less substantial.

1.0.2 The Asbestos-Carbon Nanotube Analogy

The objectives of this thesis work are motivated by the asbestos-carbon nanotube analogy, which hypothesizes that the high-aspect ratio geometry of fiber-like nanomaterials may be sufficient to drive significant toxicity. Asbestos fibers exist as a set of six naturally occurring silicate minerals in the form of long, thin fibers. The most

Crocidolite Asbestos	
Industrial Use	Home insulation, fire-resistant materials, kitchen appliances, patching and spackling compounds, vinyl sheeting and flooring, paints, and various types of textiles
Key Properties	Long and rod-like with high tensile strength, exhibit durability in both acidic and alkaline environments, and are thermally insulating
Lung Deposition	Lung bifurcations, bronchi, diameter dependent deposition
Clearance	Unsuccessful
Inflammation	Yes
Cytotoxicity	Yes
Mechanisms of Toxicity	Lysosomal damage, mitochondrial damage, redox cycling and ROS generation, DNA adduct formation, chromosomal damage, increased cell proliferation
Fibrosis Characteristics	Granulomatous lesions, fibroblastic foci, alveolar thickening

Table 1.1: Key Characteristics of Crocidolite Asbestos

toxic asbestos fibers are amphibole fibers, amosite, and crocidolite (most toxic), which are long and rod-like, exhibit high tensile strength, are durable in both acidic and alkaline environments, and are thermally insulating. Asbestos can be incorporated into consumer products for commercial use, including home insulation, fire-resistant materials, kitchen appliances, patching and spackling compounds, vinyl sheeting and flooring, paints, and various types of textiles. However, naturally occurring fibers are biopersistent within the human body after inhalation, and several case studies have linked amphibole asbestos with disease pathogenesis after 10 to 40 year latency periods. More recent research reveals that inhaled asbestos fibers deposit in the larger airways of the lung in a diameter dependent manner but have difficulty being cleared by mucociliary transport.

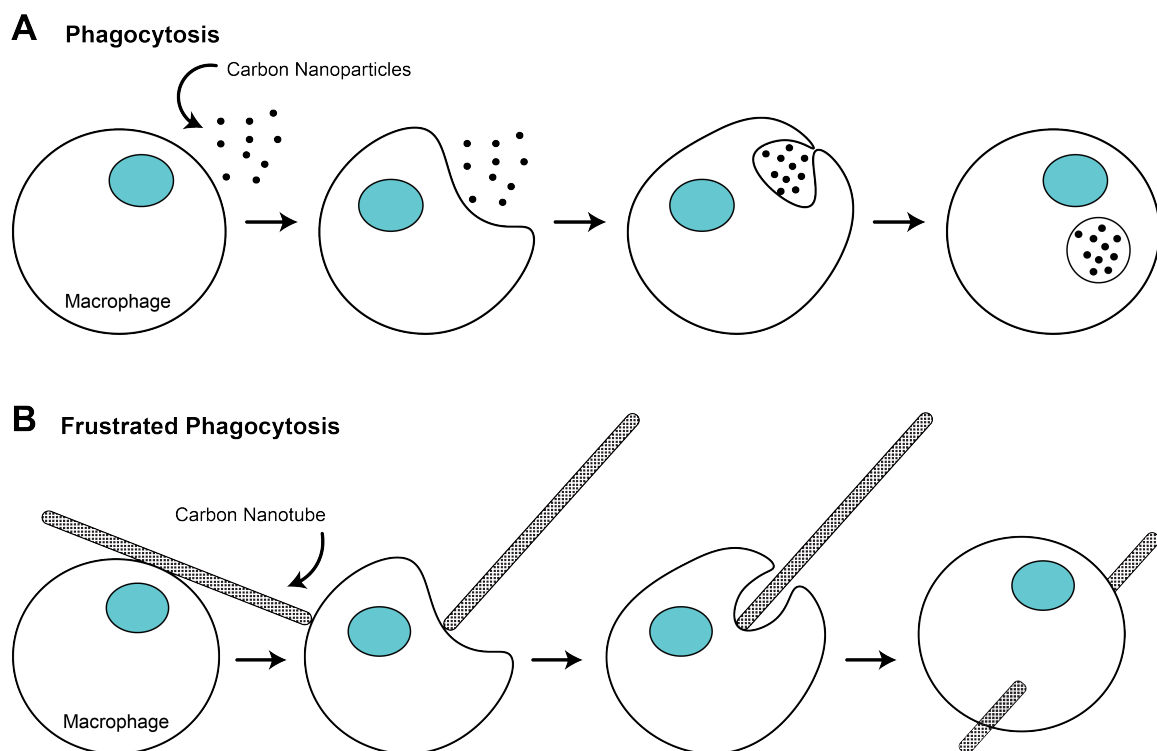


Figure 1.1: Frustrated Phagocytosis

Macrophages recruited to the site of damage will attempt to clear foreign particulates. (A) Small particulates, like isometric nanoparticles, are easily cleared through phagocytosis without inflammatory response or cytotoxicity. (B) Attempts to phagocytose large or high-aspect ratio particulates, like high-aspect ratio carbon nanotubes, can result in macrophage stress and secretion of inflammatory cytokines, leakage of intracellular contents into the extracellular space, and even macrophage death.

Over time, asbestos can reach the alveolar sacs of the lung, induce inflammation and disease [9-14], and translocate through the pleura into the pleural space [9, 15, 16]. Attempts to clear biopersistent asbestos by recruited macrophages can result in frustrated phagocytosis, defined in this context as failure to successfully phagocytose large or high aspect ratio contaminants, and increased pro-inflammatory cytokine secretion [17, 18]. Additionally, the lymphatic and circulatory systems can transport fibers from the lungs, allowing systemic circulation of asbestos fibers and deposition in other organs, sometimes resulting in a profibrotic response [9, 19]. Other cells in the lung can endocytose asbestos. As a result, asbestos can cause lysosomal damage

and release of asbestos into the cell cytoplasm where fibers interact with cellular macromolecules and organelles.

Consequently, cytoplasmic asbestos is capable of causing genotoxicity through direct interaction with DNA, causing DNA adduct formation, and interfering with mitotic spindle formation, causing chromosomal damage [15, 20, 21]. Another mechanism of action of asbestos includes the formation of reactive oxygen species (ROS). The redox activity of iron in asbestos, in conjunction with frustrated phagocytosis, results in the intracellular (leaked after lysosomal damage within cells) and extracellular release (incomplete fiber uptake and failed phagocytic vesicle formation) of reactive oxygen species including hydrogen peroxide and superoxide radical anions [22-26]. The resulting release of ROS, genotoxicity, and cytotoxicity optimizes conditions for the pathogenesis of fibrosis and mesothelioma. Crocidolite asbestos fibers have also been confirmed to be a potent epidermal growth factor receptor (EGFR) ligand, resulting in an increase in cell proliferation due to increased affinity for asbestos over EGFR's natural ligand, epidermal growth factor (EGF) [27, 28].

Physico-chemical properties shared between asbestos and carbon nanotubes include high aspect ratio, high biopersistence due to low solubility, and high tensile strength. Both types of particles are capable of interacting with biological macromolecules, including lipids, proteins, and DNA. Several human case studies detailing both the mode and mechanism of action of asbestos exist. At the same time, carbon nanotube toxicity is supported by profibrotic activity in in vitro and in vivo models. In vitro assays detail similar mechanisms of toxicity for carbon nanotubes, which include oxidative stress, stimulation of the secretion of pro-inflammatory and profibrotic mediators, and support indirect and direct genotoxicity. In vivo studies comparing asbestos and multiwall carbon nanotubes support reduced clearance, inflammation, and fibrogenesis in acute and chronic models, with some multiwall carbon nanotubes,

specifically Mitsui MWCNT-7, displaying genotoxicity and carcinogenic potential in rodent models [29-33].

1.0.3 Engineered Carbon Nanomaterials

Engineered nanomaterials (ENMs) can be classified based on their chemical composition, including carbon-based, metal-based, dendrimer, and composite nanomaterials. For the purposes of the thesis, we focus exclusively on the carbon-based nanomaterial class, choosing to evaluate pristine carbon materials that are less than 1 μm in any of three-dimensions. Three subclasses of carbon nanomaterials include nanoparticles, nanotubes, and sheet-like materials. These nanomaterials are comprised exclusively of carbon but can be functionalized to change their physical and chemical properties. Most engineered nanomaterial physicochemical properties are tuned through their production method and further processed for incorporation into specific types of products and optimized to perform specific functions. The immense number of products into which carbon nanomaterials are incorporated increases the potential for human exposure through workplace exposure or environmental exposure. Nanoparticles can also carry co-contaminants, including metal ion catalysts [34, 35], detergents or solvents that aid in the production of nanoparticles with a consistent size [36], or adsorbed contaminants from environmental release and reactivity [35, 37]. Simultaneously, novel ENMs are continuously being developed to meet growing needs for technological and pharmaceutical advancement, requiring new high-throughput assays to characterize ENM bio-distribution, mode of action, and mechanism of action to predict human health risk across all classes of carbon nanomaterials. The potential diversity in physical and chemical characteristics of countless nanomaterials presents diversified risk potential for several types of toxicities driven by changes in size, shape, stiffness, functionalization, coating, and chemical or metal co-contaminants [38-40]. Furthermore, elucidating the mechanisms of toxicity and screening to predict human

risk yields valuable information for "safer by design" initiatives to increase human safety without relinquishing valuable engineered functional properties [41].

Size and Shape Dependent Particle Deposition in the Human Lung

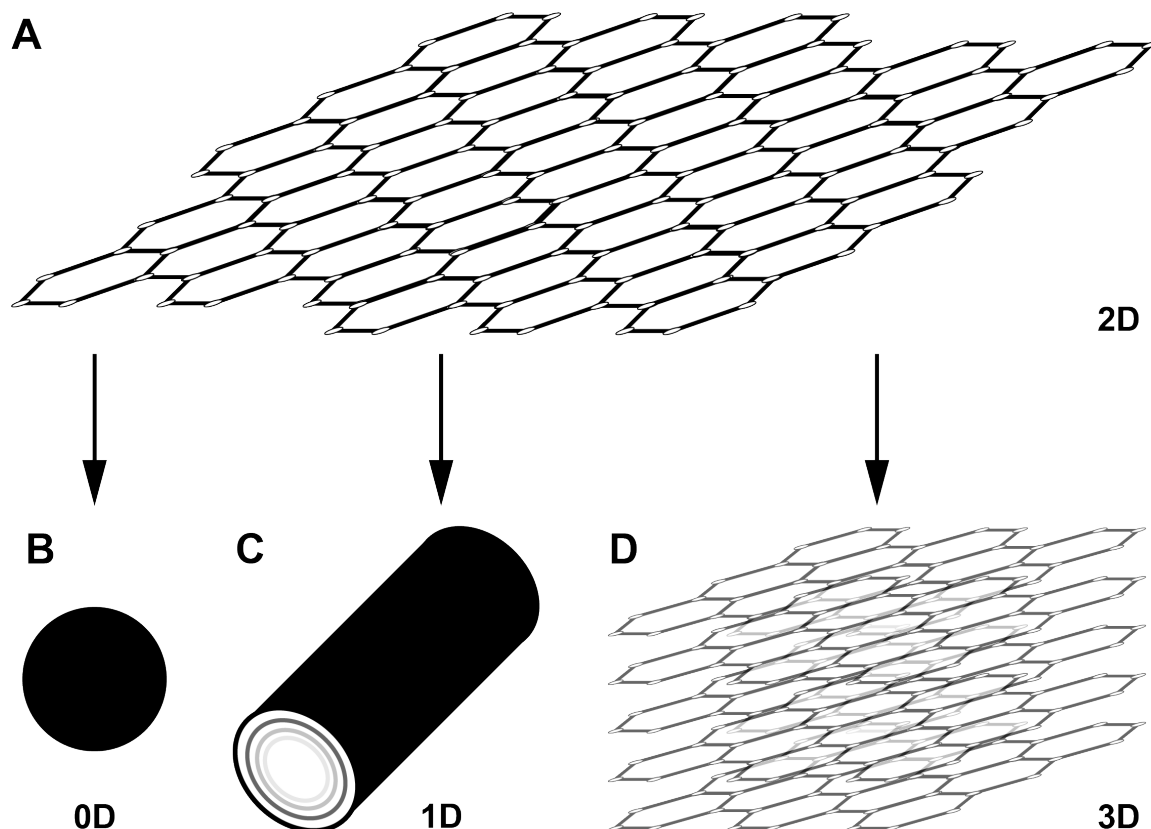


Figure 1.2: Engineered Carbon Nanomaterial Geometry

(A) Two-dimensional (2D) single plane carbon lattices are representative of single layer graphene materials. (B) Zero-dimensional (0D) carbon particles are representative of fullerenes, carbon black, and other isometric nanoparticles. (C) One-dimensional (1D) fibrous particles resemble the high-aspect ratio shape of single-wall, (N)-wall, multi-wall, and flexible nanotubes. The example depicted is best representative of a multi-wall carbon nanotube. (D) Two-dimensional carbon lattices can stack for form multi-layer (3D) materials that best represent multi-layer graphenes. Black coloration also represents arrangements of carbon lattice.

Engineered carbon nanoparticles deposit onto the lung parenchyma in a size- and shape-dependent manner and can chronically persist [42, 43]. Zero-dimensional (0D) particles describe particles of nano-size that localize their effects to a discrete location

or "point" that can deposit in the alveoli of the human lung (Figure 1.1B). Alternatively, one-dimensional (1D) fibrous particles tend to deposit at lung bifurcations in an aspect ratio-dependent manner, with higher aspect ratio particles depositing earlier in the bronchi or bronchioles in a diameter-dependent fashion (Figure 1.1C). Finally, two-dimensional (2D/3D) particles resemble sheets (Figure 1.1A) or stacks of sheets (Figure 1.1D) and deposit in the lung based on size and shape, with smaller lateral dimension materials behaving like particles, while larger sheets deposit higher in the lung. Apart from ultrafine particles, which behave according to different kinetics due to their size, all previously discussed nanoparticles have the potential to reach deep regions within the lung and cause acute or chronic inflammatory responses and perpetuate fibrotic disease [44-48].

1.0.4 Carbon Nanoparticles and The Human Lung

Manufacturing, Properties, and Toxicity of Carbon Black

Carbon black is most commonly manufactured through incomplete thermal decomposition of hydrocarbons and is used as a reinforcement additive in rubber products and is incorporated into inks, paints, and coatings. Carbon black has low solubility, is thermally conductive, and is light absorbent [49]. Carbon black is an isometric, 0D carbon nanoparticle that is often utilized as a negative particle control in in vivo and in vitro research. This is due to several case studies that support the non-toxic nature of these materials after inhalation. Human studies run by the International Agency for Research on Cancer (IARC) evaluated the carcinogenicity of carbon black and determined that carbon black inhalation does not result in any detectable excess risk of lung cancer or disease after exposure after correcting for confounding job histories and biological data, as well as smoking history [50]. In a cohort study on carbon black exposure in production workers in the U.S., increased rates of death as a result of lung cancer were not observed in those who had worked for at least 12 months in 1 of

Carbon Black	
Industrial Use	Rubber, ink, paint, coatings
Key Properties	Low solubility, thermally conductive, light absorbent, hydrophobic
Lung Deposition	Alveolar sacs
Clearance	Successful
Inflammation	None / Minimal
Cytotoxicity	None
Mechanisms of Toxicity	None
Fibrosis Characteristics	None

Table 1.2: Key Characteristics of Carbon Black

18 U.S. plants [51]. In the UK, a marginal increase in elevated lung cancer mortality was associated with two carbon black production sites but not in three others. Overall, the study observed no increase in carcinogenesis or other disease mortality [52]. This was further corroborated by another similar study in Germany [53]. Studies on the effects of carbon black in rodent models have observed no adverse effects on body weight or reproductive efficacy [54]. Historically, there have been several high concentrations of carbon black exposure in acute rodent studies that have elicited inflammatory responses thought to be related to the high surface area of carbon black. These studies have been invalidated using lower exposure concentrations and failed to reproduce the initial high concentration morbidities. No developmental toxicity or fibrogenic endpoints in humans or in in vivo models have been reported. Additional in vivo and in vitro tests have shown no significant increase in fibrotic biomarker expression and in vivo studies with chest X-ray or computerized tomography have shown particle deposits in the lung with no visible adverse effects to lung physiology [IARC Monographs Volume 93].

Manufacturing, Properties, and Use of Carbon Nanotubes

Carbon nanotubes are carbon lattices connected end-to-end to form a cylinder and possess a diameter in the nanometer range. Carbon nanotubes can be synthesized through several methods. The most common method is the catalytic deposition of hydrocarbons, but other methods include electric arc discharge and laser ablation. There are two most common types of carbon nanotubes. Single-wall carbon nanotubes (SWCNTs) consist of a singular carbon lattice rolled into a cylinder, while multiwall carbon nanotubes (MWCNTs) have thicker walls with several interior cylinders. As metal ions most often act as catalytic materials on which carbon nanotubes are grown, the purity of collected carbon nanotubes is impacted by the success of removal of metal ions. Physical and chemical characteristics linked with single-wall versus multiwall versus metal ion contamination characteristics impact the biological effects of carbon nanotubes and their toxicity [55, 56].

Additionally, characteristic physical features of carbon nanotubes include high thermal and electrical conductivity, high tensile strength, and low solubility. These key properties make carbon nanotubes ideal for incorporation into consumer products, including cosmetics, batteries, televisions, computers, nanoelectronics, and lightweight, high strength materials including those used in vehicles and protective coatings. However, flexible carbon nanotubes do exist and exhibit high bending angles that can occur with relatively low applied forces and exhibit high resistance to breakage even under high strain [57]. Incorporation of flexible carbon nanotubes into consumer products allows for the production of flexible sensors and display screens that are long-lasting with high performance overtime, even rivaling stiff carbon nanotube-containing products [58].

Mechanisms of Action of Pristine Carbon Nanotubes in the Human Lung

No case studies of human exposure to carbon nanotubes have been reported. However, pristine single-wall carbon nanotubes and pristine multiwall carbon nanotubes (MWCNTs) have been shown to form large aggregates in rodent models after pharyngeal aspiration. Mice exhibit formation of fibroblastic foci, persisting clusters of active fibroblasts and myofibroblasts, granulomatous tissue, thickening of the alveolar walls, and decreased bacterial clearance with an increased incidence of infection [59-61]. Rats exposed to purified multiwall carbon nanotubes had deposits of large MWCNT aggregates, granulomatous lesions, and alveolar wall thickening. When MWCNTs were finely ground, MWCNTs were better dispersed throughout the lung. Both intact and ground MWCNTs persisted in the lung for over two months and continued to promote inflammation and granulomatous tissue formation [55]. Mitsui MWCNT-7 exposure results in pulmonary fibrosis at one week post-exposure with inflammation, granulomatous lesions, and nanomaterial invasion into the pleura by 56 days [62, 63], with similar research also observing alveolar thickening [64] and progressive fibrotic disease out to a year [43] in mice. Exposure to Mitsui MWCNT-7 also increased Ras production in mice at eight weeks post-exposure and increased levels of matrix metalloproteinases (MMPs) and MMP inhibitors. However, it did not induce significant levels of macrophage apoptosis [65]. Changes in gene expression in the mouse indicate oxidative stress at acute time points [66].

Furthermore, gene expression changes after exposure to Mitsui MWCNT-7 are consistent between in vitro primary epithelial cells in monoculture, in vitro epithelial/endothelial cells in co-culture, and in vivo studies in mice at 56 days post-exposure [67, 68]. Gene expression studies in human epithelial BEAS-2B and human fibroblast CRL-1490 cells support that Mitsui MWCNT-7 significantly increases TGF- β 1, fibroblast growth factor-2, and MMP-9 gene expression. This effect was matched to increases in TGF- β 1, TGF- β RI, and collagen type-1 in vivo in mice [6]. Rodent and

Mitsui Multi-wall Carbon Nanotubes	
Industrial Use	Cosmetics, batteries, televisions, computers, nanoelectronics, and lightweight, high strength materials including those used in vehicles and protective coatings
Key Properties	High thermal and electrical conductivity, high tensile strength, and low solubility, hydrophobic
Lung Deposition	Lung bifurcations, bronchi, diameter dependent deposition
Clearance	Short = Successful, Long = Unsuccessful
Inflammation	Yes
Cytotoxicity	Yes
Mechanisms of Toxicity	Lysosomal damage, mitochondrial damage, ROS generation, DNA damage
Fibrosis Characteristics	Granulomatous lesions, fibroblastic foci, alveolar thickening

Table 1.3: Key Characteristics of Mitsui Multi-wall Carbon Nanotubes (MWCNT-7)

in vitro studies also support that toxicity is length-dependent. Carbon nanotubes greater than 5 μm in length have significantly higher toxicity than carbon nanotubes shorter than 5 μm . This makes short MWCNTs ideal for therapeutic use due to the increased capacity for systemic circulation and reduced toxicity at sites of deposition in the liver and kidneys [69, 70]. Mitsui MWCNT-7s are the only MWCNT designated as "potentially carcinogenic," with supporting research in rodent models treated with MWCNT concentrations extrapolated from quantified industrial levels of aerosolized nanoparticles [71, 72]. Ongoing research is still required to confirm other potential mechanisms of toxicity; however, mechanisms of action may include oxidative stress leading to DNA adduct formation, cell cycle interference, and stimulation of proliferation in any of a broad array of carbon nanotube subtypes [2, 73, 74]. The extensive research confirming asbestos-like toxicity further validates our use of Mitsui MWCNT-7 as positive control particles capable of inducing profibrotic endpoints in this thesis research.

Therapeutic Uses of Carbon Nanoparticles and Nanotubes

Both carbon nanoparticles and nanotubes are growing in popularity as drug delivery systems and biosensors for therapeutic use [70, 75, 76], with some having entered clinical trials. Proposed therapeutic uses include cancer therapy [77], gene therapy, drug delivery, vaccine delivery, bactericide and fungicide activity, and bio-imaging and live imaging functions. Carbon nanoparticles and nanotubes are also incorporated into many composite scaffolds and composite materials, some of which are deemed bio-compatible and can be used for tissue engineering and regenerative medicine [78].

Manufacturing, Properties, and Use of Pristine Graphene

Graphenes are two-dimensional sheets of carbon atoms formed into a honeycomb lattice and are a fundamental building block of all carbon allotropes. Graphene sheets have a high surface area, high thermal conductivity, high tensile strength (variable across the graphene surface), and optical transparency for graphenes with few layers. Graphene is most commonly synthesized through chemical vapor deposition methods but can also be synthesized through mechanical cleavage, arc discharge, the Hummers method (defined as chemical oxidation of graphite followed by mechanical exfoliation), or longitudinal unzipping of carbon nanotubes. The chosen synthesis protocol controls the shape and size of resulting graphene products. Graphene is a highly polar and hydrophobic nanomaterial and requires oxidation to improve dispersibility in media [79]. The most chemically active parts of graphenes are their surfaces and edges, which have been observed to aid in attachment to biological molecules and promote interaction with cell membranes. Graphene nanomaterials are also incorporated into several consumer products, including ink, barrier products and filters, composite materials that are lightweight with high tensile strength such as those used for vehicles, batteries, capacitors, and semiconductors, and electronics including televisions and phones [79-89].

Mechanisms of Action of Pristine Graphene in the Human Lung

Graphenes are a large family of 2D nanomaterials that are currently being evaluated by in vitro and in vivo methods. Graphene family nanomaterials can vary in shape, size, surface area, layer number, lateral dimension, stiffness, defect density, and purity but often exhibit sharp edges and rough surfaces that promote cell uptake of smaller graphenes over larger, smooth graphenes. The high surface area of internalized graphenes increases biological macromolecule absorbance inside the cell, with adsorbent potential being inversely related to the number of layers. Graphene family nanomaterials have been found to have anti-bacterial properties and cause cytotoxicity and genotoxicity in mammalian cells. Surface modifications on graphene and reduced graphene can decrease graphene cytotoxicity and impact graphene pharmacokinetics, increase systemic distribution, and decrease systemic toxic effects of graphene exposure for all three administration routes, intravenous, intraperitoneal, and oral, in rodent models [90-96]. Murine models of inhalation toxicity for graphenes with an average diameter of 5 μm resulted in significant recruitment of macrophages and granulocytes at 24 hours after exposure and increased production of pro-inflammatory cytokines compared to carbon black and vehicle control groups [97]. Granulomatous lesions, thickening of the alveolar wall, and reduced clearance have also been observed in graphene-exposed rodents compared to carbon black controls, suggesting an effect of particle shape on the efficacy of clearance [97, 98]. Few-layer graphene exposures in mice by intratracheal instillation resulted in acute lung injury and inflammation at four and six-week timepoints [99, 100]. Graphene nanoplatelets larger than 5 μm in lateral dimension produced inflammation in both the lung and pleural space between one and seven days post-exposure.

Graphene nanomaterials can penetrate physiological barriers and cellular structures following interaction. Sharp edges can penetrate cells by ripping through cellular membranes leading to cell death. Intranasal administration, intratracheal instillation,

Graphene	
Industrial Use	Ink, barrier products and filters, composite materials that are lightweight with high tensile strength such as those used for vehicles, batteries, capacitors, and semiconductors, and electronics including televisions and phones
Key Properties	High surface area, high thermal conductivity, high tensile strength (variable across the graphene surface), and optical transparency for graphenes with few layers, hydrophobic
Lung Deposition	Size dependent deposition
Clearance	Small – successful, Large – unsuccessful
Inflammation	Yes, size and concentration dependent
Cytotoxicity	Yes, size and concentration dependent
Mechanisms of Toxicity	Metabolic changes, lysosomal leakage, cell membrane damage, ROS generation, autophagy, necrosis
Fibrosis Characteristics	Granulomatous lesions, fibroblastic foci, alveolar thickening

Table 1.4: Key Characteristics of Graphene

and inhalation of graphenes result in decreased clearance, translocation to the liver and spleen, and cytotoxicity following endocytosis of graphene less than 100 nanometers in lateral dimension. In vitro assays have confirmed that pristine graphene exposure at concentrations between 0.01-100 $\mu\text{g/mL}$ exhibit dose- and shape-dependent cytotoxicity [101] that can be reduced with surface functionalization acting to decrease hydrophobicity [102]. Pristine and coated graphene toxicity is driven by the generation of ROS, differential expression of proteins involved in metabolic pathways, cytoskeleton interference or damage, and negative impacts on cell growth, as well as changes to levels of calcium-binding proteins [101]. Pristine graphene also activates macrophages through the depletion of mitochondrial membrane potential in tandem with ROS generation, which triggers apoptosis. Increased expression of MAP-kinase and TGF- β -related signaling pathways, as well as increases in the activity of pro-

apoptotic family members in the BCL-2 protein family [103], replicate profibrotic endpoints observed in rodent models. However, existing questions remain regarding the toxicity of graphene and graphene oxides with some research concluding that no cytotoxicity or genotoxicity can be attributed to the exposure [104]. While differing conclusions are most likely due to differences in methods and material characteristics, discrepancies only serve to highlight the need for more sensitive and reproducible research methods to test for nanomaterial toxicity.

Lysosomal Damage by Carbon Nanotubes and Graphenes

Uptake by endocytosis and phagocytosis into lysosomes is dependent on particle shape and directionality. Biopersistent large and high aspect ratio nanomaterials that can survive within lysosomes and phagolysosomes can experience prolonged contact with the lysosomal membrane, resulting in lipid extraction, transient puncturing, or destruction [105-107]. Lysosomal damage allows for the release of contents from the low pH lysosomal space that are capable of degrading proteins, damaging organelles, releasing nanomaterials into the intracellular space, and leaking of signaling factors that promote inflammasome activation and apoptosis [105, 107-111]. Escaped nanotube [112-117] and sheet-like nanomaterials [118] also can induce DNA damage by directly interacting with the nuclear and mitochondrial membranes [119-121]. As a result, lysosomal damage caused by biopersistent nanomaterial exposure can cause apoptosis and necrosis in the human lung, leading to lung cell damage and activation of the wound healing response. Finally, if biopersistent particles continue to evade clearance mechanisms, dysregulation of the wound healing response and chronic activation of wound healing cells can destroy the pulmonary architecture.

1.0.5 Pulmonary Fibrosis

Dysregulated Wound Healing in Fibrogenesis

Pulmonary fibrosis is the result of repeated acute or chronic lung damage caused by pathogenic infection, physical or chemical trauma, genetic predisposition, impaired regenerative response, and, for the purposes of this thesis work, inhalation of biopersistent particles [122, 123]. Fibrosis is a chronic, progressive, and terminal disease with increased incidence in aging individuals and patients with suppressed immune systems with no cure apart from lung transplantation. With a median age of diagnosis of 65 years, patients experience a rapid decline in the quality of life with patient survival averaging 3-5 years a lung transplant. Symptoms associated with fibrosis include shortness of breath, a dry, hacking cough, and pain caused by excessive extracellular matrix (ECM) deposition in typically high surface area regions of the human lung. Excessive ECM deposition results from impaired or dysregulated wound healing in response to damage and destroys the lung architecture over time by replacing it with scar tissue. Dysregulated wound healing is associated with unsuccessful clearance of damaged tissue and biopersistent contaminants that stimulate continued recruitment and activation of white blood cells and sustain high inflammatory cytokine levels. Inflammation and biopersistent contaminants repeatedly activate the epithelial cell – fibroblast signaling axis responsible for the control of damage-signaling, fibroblast activation and proliferation, and differentiation of multiple cell types important for successful wound healing. Inflammatory cytokines prompt the formation of fibrinous clots, across which monocytes, macrophages, and fibroblasts can migrate to reach sites of damage. Once there, fibroblasts undergo fibroblast to myofibroblast differentiation and exhibit increased cell-mediated contractility and ECM remodeling and deposition capabilities. However, dysregulated wound healing responses fail to regulate the final repair phases of wound healing, resulting in prolonged activation and increased

lifetime of recruited myofibroblasts, which continue to deposit excessive amounts of ECM that destroy lung architecture. This irreparable destruction is concomitant with the formation of granulomatous lesions and fibroblastic foci that accompany alveolar wall thickening and impaired gas exchange. Granulomatous lesions are defined as inflamed regions around granulomas. Granulomas are masses of cells formed around damaged tissue comprised of epithelioid macrophages and small numbers of other resident cell types, lymphocytes, and eventually fibroblasts and epithelial cells, that can form transiently and resolve or become persistent. Fibroblastic foci are discrete clusters of activated fibroblasts and myofibroblasts that are thought to function as discrete sites of injury and repair [122, 124-137]. During the resolution phase of wound healing, macrophage and myofibroblast apoptosis is tightly controlled and is thought to become dysregulated due to prolonged TGF- β signaling [138], leading to the stable formation of granulomas and fibroblastic foci throughout the progression of fibrosis.

Treatment Approaches for Pulmonary Fibrosis

The increasing global incidence and incurable nature of fibrosis support the need for the development of translational in vitro assays capable of adverse human health risks with dual use for drug screening. Current methods for fibrosis management and treatment are insufficient to increase the patient's quality of life after diagnosis. Commonly utilized and novel drugs alike have inconsistent efficacy between patients with similar prognoses or even in the same patient over time as the disease progresses. Currently, there are only two approved drugs regularly administered to patients to slow disease progression. Better systems of palliative care to manage the symptoms of fibrosis are being developed, but lung transplantation remains the only treatment that can improve the quality of life long-term. Pirfenidone is one of the two marketed antifibrotic drugs with proven antioxidant and anti-inflammatory activity. Pirfenidone has successfully reduced the decline in forced vital capacity and slowed disease pro-

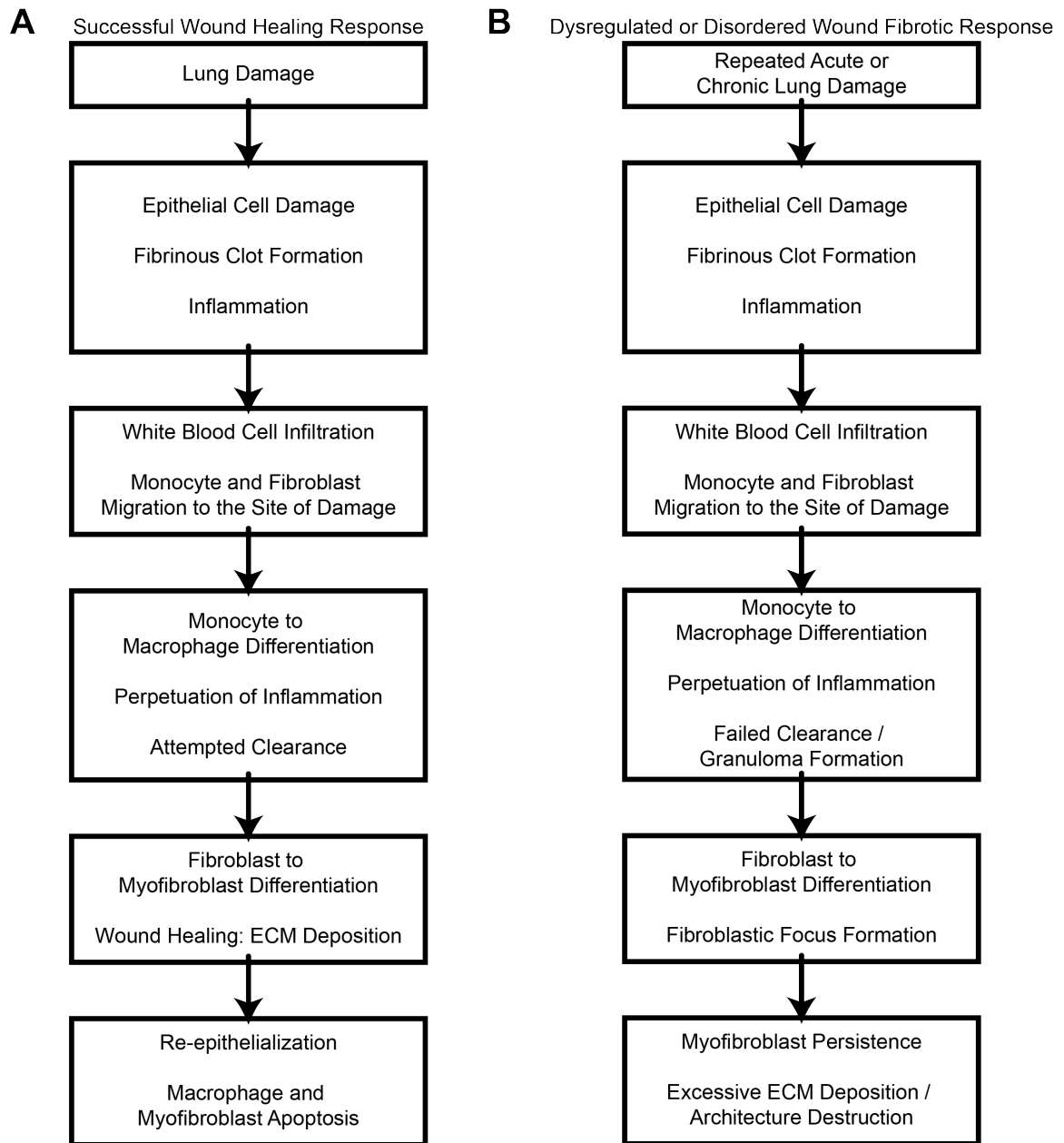


Figure 1.3: The Wound Healing Response

(A) Successful wound healing in the human lung requires several functional steps to occur in the correct order to reach and complete the repair phase of wound healing. (B) Alternatively, failure to complete these steps in the correct order or to correctly regulate secreted cytokine levels and signaling pathway activity results in failure to complete the repair phase. Dysregulated repeated acute or chronic wound healing destroys the pulmonary architecture and promotes fibrogenesis.

gression in some patients compared to placebo groups in clinical trials [139]. In vitro and rodent models, Pirfenidone acts upon multiple pulmonary cell types to decrease secretion of and response to TGF- β ligands. TGF- β pathway inhibition by Pirfenidone decreases fibroblast proliferation, activity in the epithelial cell-fibroblast axis to decrease secretion of profibrotic cytokines, and accumulation of ECM associated with tissue destruction [140-147]. However, Pirfenidone has variable success in improving patient survival in clinical settings [133, 148-152]. Pirfenidone is often administered with Nintedanib, a tyrosine kinase inhibitor that boasts inhibitory effects on fibroblast proliferation and fibroblast to myofibroblast differentiation [153]. Simultaneous treatment with Nintedanib enhances survival with significant decreases to TGF- β signaling and autophagy processes, although the exact mechanism remains poorly understood [149, 154]. The efficacy of both drugs to slow fibrosis progression in patients has been noted in several clinical trials. However, even with combinatorial administration proving to be most effective, some patients garner no beneficial effects [155-157]. A number of comorbidities associated with pulmonary fibrosis have the potential to worsen disease prognosis and are not addressed by treatment with Pirfenidone and Nintedanib [158, 159]. Novel, experimental patient treatments attempt to target drivers of fibrosis, including ECM remodeling enzymes, inflammatory cytokines, and growth hormones. While palliative care can treat fibrosis symptoms including increased mucus production and viscosity, pulmonary hypertension, anxiety and depression, and nausea and vomiting associated with Pirfenidone and Nintedanib regimens, small molecule and antibody-based drugs are being tested for efficacy against biomarkers that are heavily associated with progression of fibrosis. An extensive list of compounds, antibodies, and small-molecule inhibitors have been proposed to treat fibrosis and are summarized by Duffield et al. While several of the listed treatments have failed in clinical trials, more therapeutics targeting similar pathways are in clinical trials [160]. With no long-term options for the effective treat-

ment of fibrosis, the best way to protect people at risk is a prophylactic approach. By developing and utilizing sensitive assays to predict the profibrotic potential of untested and novel nanoparticles, better regulations can be implemented to protect employees in the workplace from being exposed to fibrogenic airborne contaminants. In order to address this need, research objectives for this thesis work incorporate features including TGF- β signaling pathway biomarkers of fibroblast activation and fibroblast to myofibroblast differentiation, co-culture work evaluating the epithelial cell-fibroblast axis, and changes to profibrotic gene expression and protein abundance to predict the fibrogenic potential of a panel of particle exposures.

1.0.6 The TGF- β Signaling Pathway

The Canonical TGF- β Pathway

Several key pathways essential for human lung development reactivate during wound healing, arguably the most important being the TGF- β pathway. Cells actively expressing TGF- β 1, a potent the TGF- β pathway ligand, in the adult human lung include the bronchial epithelium, mesenchymal and endothelial cells, and alveolar macrophages with the presence of TGF- β receptor ligands correlating with disease severity and patient survival. Furthermore, TGF- β 1 secretion acts as a chemoattractant to recruit fibroblasts to sites of damage, spur myofibroblast differentiation, and prevent degradation and promote deposition of ECM [161]. These pathologically relevant and biologically essential functions of the TGF- β pathway make targets of the TGF- β pathway ideal biomarkers for measuring changes in human lung cell populations for the development of in vitro assays.

The TGF- β family consists of three ligand isoforms (TGF- β 1, 2, and 3), three receptors (TGF- β RI, II, and III), and several signaling mediators termed SMADs. TGF- β ligands are secreted by all responding cell types in the fibrotic lung and can be released from the ECM by the mechanical or enzyme-driven release of latent TGF- β 1

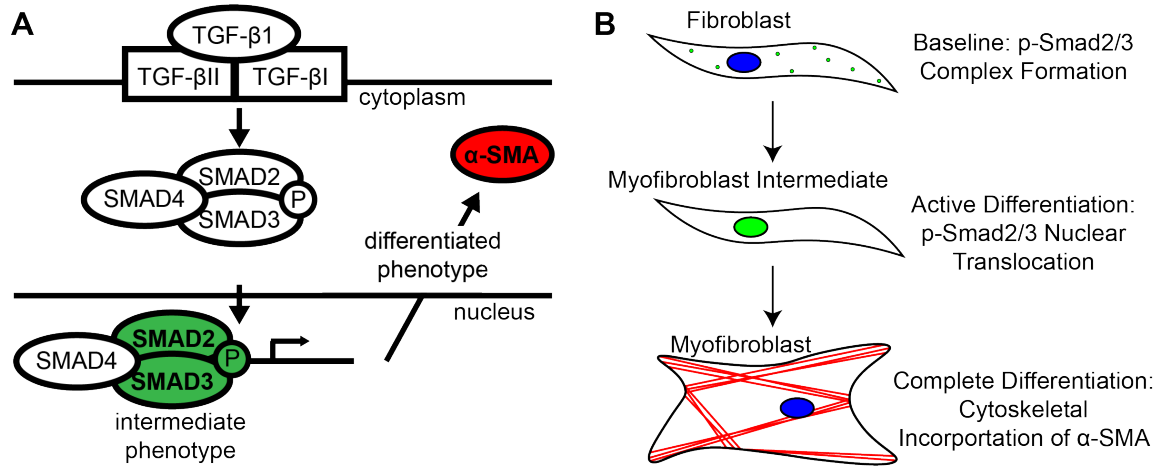


Figure 1.4: The TGF- β Signaling Pathway

(A) The TGF- β signaling pathway can be activated by the binding of TGF- β 1 to TGF- β receptors. Smad2 and Smad3 proteins form a phosphorylated complex that can translocate into the nucleus, where it acts as a transcription factor to increase expression of myofibroblast genes. α -SMA is then incorporated into the myofibroblast cytoskeleton. (B) The application of biomarker labels in the nucleus and cytoskeleton can identify myofibroblast intermediate and myofibroblast phenotypes, respectively.

from latent TGF- β complexes. TGF- β 1 and TGF- β 2 have similar biological activities in in vitro experiments, but TGF- β 1 has the most potent effects in in vivo studies. Ligand-bound TGF- β receptors heterodimerize and prompt Smad-2 and Smad-3 complex formation. The Smad2/3 complex becomes phosphorylated and binds Smad-4 before translocating into the nucleus. There, the complex acts as a transcription factor to regulate the expression of ECM and ECM remodeling proteins, proliferation, differentiation, or apoptosis-associated genes [162-165]. Matched SWCNT and MWCNT exposure studies show that fibers greater than 5 μ m in length significantly increased the expression and deposition of collagen type 1, increased expression of TGF- β R1, increased Smad2 phosphorylation, and increased TGF- β 1 expression and secretion [166]. TGF- β 1 is secreted in a latent form, bound to the latency-associated peptide (LAP). Alveolar epithelial cells in fibrotic microenvironments increase expression of the integrin α 6 (dependent on FAK signaling and interaction with fibronectin

or fibrin), which binds LAP and converts TGF- β 1 into an active form and promotes EMT [167] or induces apoptosis [168, 169].

Several in vitro studies evaluating the epithelial cell/fibroblast signaling axis support that MWCNTs also stimulate increased expression and secretion of TGF- β 1 and lead to activation of the TGF- β signaling pathway and promote EMT and myofibroblast differentiation and is dependent on both length and concentration [170]. Activation of the TGF- β signaling pathway results in the formation of p-Smad2/3 complexes that enter the nucleus where the complex acts as a transcription factor to increase myofibroblast and mesenchymal cell gene expression profiles. Resident epithelial cells can undergo differentiation to a fibroblast-like phenotype characterized by increased α -SMA and vimentin and decreased E-cadherin and other junctional protein gene expression. Fibroblasts and macrophages also increase gene expression and secretion of TGF- β 1, with fibroblasts increasing gene expression of collagen type 1 and 3 and MMP family proteins [171].

Latent TGF- β Activity

Latent TGF- β complexes form when TGF- β covalently binds to a TGF- β pro-peptide called a latency-associated peptide (LAP) via disulfide bonds. LAP is cleaved from mature TGF- β precursors in the Golgi, but LAP and TGF- β remain strongly bound through non-covalent interactions. LAP and TGF- β form large latent complexes that can be activated by integrin binding and cell-mediated contraction. Latent TGF- β complexes act independently of intracellular activity by stimulating the stabilization of microfibril bundles and regulating elastic fiber assembly in lung cells, predominantly fibroblasts [172]. TGF- β incorporated into latent complexes associated with fibronectin is capable of interacting with cells before or after mechanical or enzymatic intervention, making it free to drive proliferation, migration, differentiation,

ECM deposition, and contractility of resident lung cells, most notably epithelial cells and fibroblasts [173].

While TGF- β 1 and TGF- β 2 are the most active in the human lung, but the most common TGF- β isomers found within latent complexes are TGF- β 1 and TGF- β 3. Latent forms of TGF- β are 200 times less potent than free TGF- β [174]. TGF- β 1 and TGF- β 3 within latent complexes are recognized by alpha five integrins with loss of latent TGF β complexes resulting in multiorgan inflammation and defects in vasculogenesis [175]. Stiffening of human lung tissue during fibrogenesis increases the mechanical stress on resident fibroblasts. Fibroblasts activated by TGF- β 1 and rising mechanical stress to undergo differentiation into contractile myofibroblasts can exert force to release TGF- β from latent complexes. The release of TGF- β 1 from latent complexes directly increases activation of downstream Smad targets, suggesting the potential for integrin-mediated control of myofibroblast activity as a treatment for fibrosis [176-178].

Fibroblast to Myofibroblast Differentiation Checkpoints

The Smad signaling family consists of several effector and repressor proteins that control the activation of the TGF- β 1 signaling pathway. Genetic models of overexpression of Smad3, activated by TGF- β 1, increases α -SMA expression that is directly repressed by Smad7 [179]. Fibroblasts and myofibroblasts can exert mechanical forces to release latent TGF- β 1 embedded within latent complexes incorporated into the lung stroma. This is also true in in vitro models using any type of ECM scaffold or where cells may lay down matrix. Regulation of the mechanical forces generated by fibroblasts and myofibroblasts is controlled, in part, by activation of the ROCK/Rho pathway and is linked with activation of the Smad signaling pathway, directly demonstrated through increased expression of RhoA, RhoC, ROCK1, Smad2, and MMP family members after TGF- β 1 activation [180]. When inhibited, the ROCK pathway inhibits fibrob-

last to myofibroblast differentiation through the prevention of α -SMA incorporation into the cell cytoskeleton and is regulated by the activation of serum response factor (SRF) by both mDia2 (through binding) and RhoA (through control of SRF localization) [181, 182]. mDia2 must also localize to actin stress fibers, stabilizing the microtubule polymerization required for cytoskeletal reorganization during fibroblast to myofibroblast differentiation and resulting in the increased cell-mediated contractility exhibited by myofibroblasts. Microtubule stabilization by paclitaxel attenuates TGF- β 1-induced fibroblast to myofibroblast differentiation [183-185]. In the context of this thesis work, it is possible that intermediate phenotypes of fibroblasts exist as a result of the additional control of differentiation through mDia2 and Rho family recruitment and leads to the use of intermediate transcription factor and myofibroblast biomarker endpoints to evaluate the difference between fibroblast activation versus myofibroblast differentiation.

Non-canonical TGF- β Pathway

Non-canonical activation of fibroblasts can activate the TGF- β pathway in the absence of traditional TGF- β ligand binding. Cathepsin B can activate fibroblasts through the regulation of cystatin C secretion, promoting inhibition of cathepsin degradation and increasing p-Smad2/3 complex formation and phosphorylation without activating MAP-kinase or JNK pathways. This pathway has been characterized in liver cells to promote epithelial to stellate cell differentiation in liver fibrosis but may be a viable pathway for fibroblast to myofibroblast differentiation in the human lung after particle exposure [186].

1.1 Summary and Specific Aims

At present, toxicity screening relies on rodent models and a variety of in vitro models that attempt to recapitulate lung homeostasis and detect indicators of fibrogenesis, with only a subset of the in vitro models developed to assess nanoparticle toxicity. Moreover, even fewer in vitro models can detect nanomaterial toxicity at concentrations that are relevant to human exposures in the workplace or the environment [187-190]. Regulatory and drug screening purposes often require the use of rodents, increasing cost, difficulties with human translational efficacy, and a decreased ability to ask scientific questions targeting specific cell types and spatiotemporal endpoints of fibrogenesis. To address these difficulties, this thesis aims to develop and characterize several models in the context of pulmonary fibrosis to address questions about the cell-specific effects of nanomaterial exposure at acute time points. These models offer a reproducible, cost-effective, readily available materials alternative to in vivo research that integrates the study of well-characterized and emerging 2D and 3D technologies and well-characterized and emerging nanoparticle exposures at sub-cytotoxic and low concentrations. From past work in the lab, we found that 3D models of granuloma-like systems could recapitulate known human fibrotic endpoints. This research aims to de-couple facets of these previously developed complex systems to answer new questions about the effects of low concentrations of nanomaterials and their ability to drive fibrogenesis. As previously noted, several case studies have characterized the effects of inhaled, non-toxic carbon black and carcinogenic crocidolite asbestos, with several rodent models having characterized the toxic and potentially carcinogenic effects of MWCNTs. In this thesis research, we use these well-characterized particles as particle controls, in addition to comparisons between untreated and TGF- β 1-treated cell populations, to assess changes in our models driven by nanomaterials of different shape, size, and stiffness. To evaluate the effects of nanomaterials to drive fibrotic

endpoints and to demonstrate the efficacy of our models, we propose the following aims.

Aim One: Evaluate changes to p-Smad2/3 and α -SMA biomarkers of fibroblast to myofibroblast differentiation as a result of particle exposure on traditional tissue culture plastic. Hypothesis: Cells cultured on rigid tissue culture plastic exhibit reduced sensitivity for cell-particle recognition, since adhesions are dominated by the extreme differences in substrate rigidity relative to healthy tissues.

Aim Two: Quantify changes to two biomarkers of fibroblasts to myofibroblast differentiation as a result of particle exposure on tunable, soft polyacrylamide substrates. Hypothesis: Cells cultured on soft polyacrylamide substrates (comparable to healthy tissues) exhibit increased sensitivity for cell-particle recognition, since adhesions are not saturated by substrate stiffness, permitting the cytoskeletal reorganization required for fibroblast to myofibroblast differentiation.

Aim Three: Develop and characterize a 3D model of a fibroblastic focus and quantify the extent to which particle exposure drives fibroblast to myofibroblast differentiation within the system, evaluate cell-cell and cell-nanomaterial interactions by staining for cell sorting and morphological changes, and benchmark the model to known fibrogenic endpoints by evaluating profibrotic gene expression and cytokine secretion after particle exposure. Hypothesis: A 3D, scaffold-free platform of fibroblasts will detect fibroblast to myofibroblast differentiation of fibroblasts populations and overall increases in TGF- β 1 secretion after exposure to stiff fibrous particles but not non-toxic carbon black particles.

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Chapter 2

Fibroblast-to-Myofibroblast Differentiation after Carbon Nanomaterial Exposure on Compliant Substrates.

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2.1 Abstract

Toxicologists experience challenges evaluating sub-cytotoxic toxicant exposures. We have developed a single-cell screening platform capable of evaluating high-content confocal images to quantify single-cell, sub-cytotoxic phenotypic changes driven by

carbon nanomaterial exposure. We hypothesized that dysregulation of fibroblasts, wound-healing cells responsible for excessive ECM deposition in fibrosis, could be driven in part by the mechanical stimuli associated with stiff, pristine carbon particles and that this response is masked by traditional rigid cell culture techniques. Through segmented image and multivariable regression analyses, we identified single-cell features predictive of phenotypic change driven by carbon nanotubes, but not non-toxic carbon black or nano-graphenes. Soft substrate culture marginally decreases overall fibroblast heterogeneity, sensitizes fibroblasts to carbon nanotubes, and groups graphene exposures together, while fibroblasts exposed to nanomaterials on rigid substrates have similar exposure signatures. Multi-variable FAMD plotting identifies a greater spread of soft substrate cell phenotypes associated with carbon nanotube and graphene exposures, quantifying single-cell sensitivity to nanomaterial exposure typically unquantifiable without single-cell omics. Finally, we identified predictive phenotypic features through correlation and feature reduction analyses, highlighting several significant soft substrate feature relationships. These analysis platforms are universally useful for toxicologists to identify predictive features of sub-cytotoxic and toxic effects on any cell type. This universally relevant platform can also be used to evaluate the phenotypes of collected primary cells, stem cells, cancer-associated fibroblasts, etc. This work is currently being adapted to analyze monoculture and co-culture 3D in vitro microtissue models of disease for nanomaterial toxicity screening.

2.2 Introduction

2.2.1 Human Lung Composition

Several terms describe the subsets of the organization within the human lung. Tissue stroma is the extracellular matrix (ECM) scaffold of the lung, including resident cells, and is comprised of elastin, collagen, and fibronectin, among many other types

of matrix proteins [1]. The pulmonary interstitium is defined as the set of structural scaffold subtypes, not including resident cells, that are referred to as the alveolar epithelium, endothelium, basement membranes, and vascular and lymphatic tissues. Embedded signaling factors in the interstitium guide airway organization during development, contribute to pulmonary homeostasis and guide the regeneration of the stroma during wound healing [1-3]. Furthermore, maintenance of the interstitium is predominantly controlled through the turnover, remodeling, and deposition of collagen, the second most prevalent fibrous ECM protein in the human lung. However, the lung is susceptible to collagen loss as a result of aging or pathogenesis, resulting in a decrease of the collagen to elastin ratio over time [4-6] as characterized by decreased collagen gene expression and a decrease from 16% to 10% collagen density in the interstitium by 80 years of age [7-9]. Alternatively, elastin proteins have high stability and an annual turnover rate of less than 1%. The loss of collagen density in the interstitium is thought to be responsible for the decline in successful wound healing over time and the direct correlation between the prevalence of fibrosis with age. Therefore, diminished biological cues received during cell-ECM interactions can continue to drive changes in collagen abundance and fail to inform successful wound healing progression [10-12].

Density and Organization of Collagen in the Human Lung

Currently, there are 19 recognized collagen proteins. Each collagen sub-type has differential regulation at the transcriptional and translational levels with organ- and type-dependent rates of turnover. Differences in collagen synthesis are tissue-specific with different tissues exhibiting distinct levels of collagen sub-type gene expression and integration into the tissue stroma, with collagen I, III, IV, and V being the predominant collagen subtypes found throughout the bronchiolar and alveolar regions

of the human lung. In short, organ-specific cell-ECM interactions are driven by different densities and types of collagen signaling.

Maintenance of the Lung Interstitium

The pulmonary interstitium maintains the structural integrity of the human lung. The interstitium is populated by interstitial cells, including fibroblasts and pericytes, as well as macrophages, epithelial cells, and other resident cell types. Fibroblasts are responsible for synthesizing nascent ECM components and integrating them as needed to maintain lung interstitium homeostasis. Collagen, fibronectin, and laminin are critical ECM components and are commonly used as coatings in in vitro lung microenvironments. Several studies have assessed the differential effects of cell culture on collagen, fibronectin, and laminin coatings using epithelial, fibroblast, and endothelial cells. This research revealed characteristic cell organization and integrin-mediated cell-matrix interactions driven by 1) the presence and density of ECM binding sites and 2) the expression of integrin subtypes that mediate cell adherence, proliferation, and migration [4, 10, 11]. Another key signaling factor in the interstitial matrix is the TGF- β 1 incorporated into latent complexes. Latent complexes are associated with fibronectin and are capable of interacting with cells before or after mechanical or enzymatic release, making it free to drive the proliferation, migration, differentiation, ECM deposition, and contractility of resident lung cells, most notably epithelial cells and fibroblasts [12].

2.2.2 Matrix Stiffness

Healthy tissues within the human body have characteristic stiffnesses that vary by several orders of magnitude. The healthy lung has a stiffness of 2-4 kPa, increasing to between 18-100 kPa in patients with fibrosis [13]. Alternatively, aberrant stiffening is a consistent marker of disease pathogenesis, for example, in cases of cardiovascular

disease, fibrosis, and carcinogenesis [14-17]. Due to dysregulated ECM remodeling, the increase in the collagen to elastin ratio correlates with an increase in lung tissue stiffness [4-6, 18-20]. The differences between the mechanical properties of spatially distant regions in the lung are thought to drive site-specific molecular fibrogenesis. However, more research is needed to understand how the mechanical properties of the lung control homeostatic cell proliferation, gene expression, and differentiation and how best to utilize those endpoints to detect aberrant cell activity and predict fibrogenesis [21]. Mechanobiology is a field at the interface of biophysics and bioengineering that focuses on how cell and tissue mechanics and physical forces influence cell behavior, cell morphogenesis, and disease pathogenesis. When a cell recognizes a mechanical stimulus, a process termed mechanical transduction translates the mechanical signal into a physical or biochemical response. Cells have two versions of mechanosensing, outside-in and inside-out signaling, that are mediated by clusters of integrins that drive cell-matrix adhesion. Outside-in signaling is the recognition of mechanical cues in the microenvironment that affecting cellular processes such as migration, growth, and differentiation. Inside-out signaling alters the mechanical properties of the microenvironment through the exertion of traction forces and remodeling of ECM. Actin-myosin contractility is defined as the contractile activity of the cytoskeleton and is responsible for inside-out signaling at cell-matrix adhesions. Understanding complex interactions between cells and their microenvironment is important for applications in mechanobiology and tissue engineering but also the evaluation of mechanosensing as a driver of pathology [22]. The need to understand the importance of these concepts as mediators of tissue homeostasis and as potential drug targets has led to the creation of the field of Mechanomics [23].

Tissues are viscoelastic and are impacted continuously by cellular and functional forces, lung expansion and cardiac contraction, for example. Several platforms have been developed to evaluate the cell mechanome, including the application of sev-

eral ECM protein coatings, tunable substrates, soft bio-compatible substrates, soft-patterned lithography platforms, and other microfabrication techniques that allow for micromanipulation of various physical parameters that impact cell behavior [24-32]. These biocompatible substrates can mimic healthy versus pathologically stiff conditions and can be employed to research phenotypic changes driven by extracellular matrix composition and substrate stiffness.

Research using these platforms supports that soft substrates can cause apoptosis, growth arrest, decreased mature focal adhesion count, and protect against fibrogenesis. In contrast, rigid substrates influence cell phenotypes through increased substrate adhesiveness, cell stiffness, applied cell tension [25, 33-38], in addition to increased proliferation, spreading, and gradient-directed migration [37, 39]. Furthermore, fibroblasts and epithelial cells are capable of phenotypic change when switched between rigid and soft substrates [40-45], but mechanical memory has also been observed [46-49]. Fibroblasts are capable of sensing stiffening ECM over short distances, activating fibroblast populations to proliferate and differentiate even in soft microenvironments [13, 34, 50]. While some research suggests that this distance is roughly the width of an adjacent cell [51], other research suggests that fibroblasts and endothelial cells can sense as far as 100 μm away from their current location. As a result, pathologically stiff regions throughout tissues have the potential to drive fibrogenic activity in spatially distant regions. Finally, understanding substrate-driven phenotypic plasticity has been indispensable in the field of stem cell differentiation [52-62] and the study of resident and circulating stem-like cell differentiation.

The Rho/ROCK pathway is a critical mechanical transduction pathway important for the recognition of ECM stiffness, requiring both Rho A and Rho GTPase recruitment. 2D and 3D in vitro research support that cell invasion and migration is heavily linked with activation and function of the Rho/ROCK pathway. Rho A is predominantly associated with cell-mediated contractility, while Rho GTPases interact

with mature actin fibers and focal adhesion complexes and exhibit critical phosphorylation activity. Cell stiffness is regulated by the degree of actin crosslinking and bundling, which increases with increasing substrate stiffness. Fibroblasts adapt their stiffness by altering the organization and stability of microtubules that are crucial for controlling cell morphology and spreading [63, 64]. Cytoskeletal proteins are a large protein family classified into five different classes based on self-assembly behavior that aid cell stiffening. Vimentin and α -SMA interact with actin, microtubules, focal adhesions, and integrins, thus stabilizing adhesion strength [65]. Calcium-regulated actin stress fiber formation controls short-range contractile function, on the order of approximately 400 nm, and the recognition of substrate stiffness by myofibroblasts. Finally, biomaterial cues can regulate MAP-kinase, WNT, YAP/TAZ, and other pathways that also direct cell adhesion, migration, proliferation, and differentiation [66]. However, while our scientific understanding of the control of cell response to tissue stiffness is growing, further research is required to evaluate the impact of inhaled, non-physiologically stiff particles.

2.2.3 Modeling the Human Lung

There is species-to-species variation in lung and alveolar size, stromal density, relative cell count, and ECM composition. Rats, a common rodent model for evaluating fibrogenesis, are characterized to have 25-fold the number of alveoli measuring 4-fold smaller than human alveoli [67-69]. Collagen and elastin densities in the rat are also 4-fold higher [70, 71]. Finally, the percent area of the lung comprised of endothelial cells is lowest in humans compared to rat and baboon lung; however, the fraction of alveolar epithelial cells is comparable across species [67]. As a potential source of fibroblasts, controlling for differences in the number of endothelial cells and mesenchymal cells to understand fibrogenesis across species is challenging; however, translational research on alveolar epithelial cell toxicity may be more reliable.

Differences in these characteristics act as confounding variables that make human extrapolations more difficult. Moreover, in vitro methods that apply biocompatible, tunable, tissue-mimicking substrates offer alternatives to study cell-cell relationships in a more human-like environment.

Another approach to mimicking the lung microenvironment is through the use of decellularized ECM. As human decellularized ECM is not a readily available resource, decellularized porcine ECM is increasing in popularity as an alternative to rat- or bovine-derived or synthetic ECM proteins. Decellularized porcine ECM supports that porcine tissues have substantially more residual protein compared to human tissue samples on a per gram basis. Additionally, decellularized porcine tissue retains collagen type II, V, and VII proteins not found in decellularized human tissues. Alternatively, decellularized human tissue is capable of retaining collagen XII, different fibulin sub-type proteins, heparan sulfate, and periostin not found in porcine tissues [72, 73]. In addition to stiffness cues, several proteins are embedded in the lung ECM that act promote cell localization and function. For example, tenascin C, a glycoprotein integrated into the stroma during development, is associated with epithelial cell growth and resident cell differentiation and exhibits increased integration into the ECM in lung samples evaluated after wound healing and fibrogenesis [74]. Lysyl oxidase, an embedded enzyme, stabilizes collagen and elastin assembly through crosslinking activity, controlling organization and density of the ECM [75]. Additional remodeling activity is regulated by matrix metalloproteinases (MMPs) that control the density and turnover of the ECM. The MMP family is a diverse but specialized family of 11 enzymes, including three collagenases, two gelatinases, and an elastase, among many other types. Expression of the MMP family is tightly regulated at the level of gene transcription, and secreted MMPs are activated through cleavage and regulated by additional secretion of tissue inhibitors of metallic proteinases (TIMPs) [76]. Overall, as our understanding of the complexities of human lung tissue and the

pathogenesis of fibrosis grows, the scientific community will work toward determining the minimum number of biological features and interactions required to recapitulate specific aspects of homeostatic and fibrotic lung conditions accurately.

Using Tunable Cell Culture Substrates to Model Fibrogenesis

Hydrogels are defined as three-dimensional solids derived from the crosslinking of hydrophilic polymer chains. Many hydrogels are biocompatible and allow for gas, nutrient, and waste exchange, making them ideal for use in tissue engineering and regenerative medicine. Hydrogels have allowed scientists to evaluate the impacts of ECM and soluble factors on cell differentiation, cell-cell interactions, and cell-matrix interactions with variable stiffness and binding site interactions. ECM proteins and non-adherent components can be incorporated into hydrogel scaffolds to control polymerization and impact hydrogel pore size and stiffness with different adhesive and biocompatible properties [77].

2D and 3D in vitro models can incorporate bioengineered hydrogels, including, but not limited to, collagen or decellularized ECM gels, Matrigel, polydimethylsiloxane (PDMS), and polyacrylamide. Collagen and decellularized ECM gels offer cells an abundance of integrin-binding and enzyme-cleavable sites with the potential for cell culture on or within gels of varying concentration and stiffness. Matrigel is commonly used in 3D spheroid research and can be mixed with ECM proteins to increase the density of cell binding sites. However, Matrigel substrates have low stiffness, are animal-derived, and suffer from batch-to-batch variability. PDMS substrates are biocompatible, cost-effective, and easy to produce with moderately tunable stiffness, but can exhibit inconsistencies in crosslinking throughout the substrate. Polyacrylamide gels are cheap and easy to produce with two critical features that allow for the separation of chemical and mechanical signaling. Polyacrylamide gels are inert as adhesive surfaces and require an extracellular matrix protein coating for cell ad-

hesion. Additionally, polyacrylamide gels have a highly tunable stiffness that can be varied over a wide range by tuning the ratio of bis-acrylamide to acrylamide [78-80]. Polyacrylamide formulation, polymerization temperature, gelation time, and storage duration are additional features of polyacrylamide substrate fabrication that impact gel stiffness [81]. Each type of substrate is characterized by different shear stress, strain, and Young’s modulus properties that can regulate biological mechanotransduction [80]. As a result, different physical and chemical characteristics of different substrates offer unique opportunities to ask specific questions about biological systems that contribute to the complexity of fibrotic disease.

Despite the availability of several biocompatible model substrates, there is an inherent difficulty in quantifying the stiffness of the human lung due to varied organization of the lung physiology and the spatiotemporal variability associated with the stages of fibrosis. Additionally, several, but not all, physical and biochemical changes to lung cell types can be recapitulated on “infinitely” rigid plastic or glass substrates, while other behaviors can only be quantified on soft substrates. 2D in vitro models using polyacrylamide gels support that stiffness contributes to cell differentiation, but cell phenotypes differ depending on cell type and confluence. For example, confluent endothelial cells on plastic substrates can exhibit indistinguishable morphologies from those on soft substrates at low confluence [82]. More importantly for this thesis research, confluent fibroblasts are less receptive to TGF- β 1 exposure, resistant to myofibroblast differentiation [83], and are capable of recognizing substrates up to 100 μ m away [84, 85]. At present, the effects of cell confluence and cell-cell interactions on fibroblast to myofibroblast differentiation in 2D and 3D in vitro models are poorly understood and require more research to benchmark fibroblast and myofibroblast culture to known human endpoints.

2.2.4 Sources of Fibroblast-like, Fibroblast, and Myofibroblast Cells

Scientific literature reports fibroblast population heterogeneity in vivo and in vitro observed in primary cells and cell lines. Fibroblasts are ubiquitous mesenchymal cells that are active in routine tissue repair. They are most well known for their activity in fibrotic conditions in the lung, liver, kidney, and skeletal muscle, as well as activity in tissue sclerosis, heart damage, and tumor formation. Fibroblast activation and fibroblast to myofibroblast differentiation in vivo can occur in a variety of precursor cells and is dependent on several physiological and pathological events. Myofibroblast progenitors include resident fibroblasts, circulating fibrocytes, smooth muscle cells, pericytes, endothelial cells, epithelial cells, and organ-specific populations of cardiac stromal cells or hepatic stellate cells, among others [86, 87]. Currently evaluated markers of fibroblast heterogeneity include single-cell gene expression, cytoskeletal organization, and morphology [88-90], as well as increased expression of α -SMA correlating with increased sensitivity to TGF- β 1 [89, 91]. More research is required to understand if population heterogeneity stems from individual cell variability in response to chemical or mechanical cues or other factors.

2.2.5 High Content Imaging and Image Analysis

High content imaging allows for the collection of information across large-scale experiments with increased detail and attached metadata. The data contained within high content images can be evaluated using morphological, intensity, and state-of-the-art texture analysis. Image analysis profiles are useful in identifying obvious and less apparent features that may be indicative of gene expression, permanent and transient phenotypic change, and population heterogeneity, with the ability to incorporate any number of DNA, RNA, or protein biomarkers [92-96]. The ability to derive many

types of data from each image results in a data format called multivariate. Multivariate describes the presence of several features or traits that characterize each “object,” be it a population, many single-cells, or many intracellular objects like organelles. A multivariate dataset describes this set of traits as multi-dimensional relationships that allow for any given object to be classified or compared to its counterparts using multivariable regression. The results of multivariable regression describe the value of each variable to predict and classify each object. The corresponding value of each variable can be compared to select the most valuable and descriptive variables to eliminate less descriptive variables from the dataset. This systematic review of the best features to describe the dataset to perform dimension reduction is becoming more commonplace in multivariable, big data analyses. However, attempted dimension reduction results in reduced discrimination between conditions as predicted by best-fit, multivariable regressions. Therefore, the more variables that can be quantified from original images, the better the possible predictive capacity of the experiment. Steps to preprocess data include (1) a correction step, (2) distribution transformation, (3) parameter correlation-based dimension reduction, and (4) cell-to-well aggregation [97].

Image Segmentation and Morphological Analysis

Morphological analysis can evaluate the shape of whole cells or subcellular components and has proved useful in identifying the nuclear size, shape, and count abnormalities that can indicate nuclear damage [98]. Morphological features have also been used to indicate changes in cytoskeletal organization associated with epithelial to mesenchymal transition (EMT) and fibroblast to myofibroblast differentiation in pathologies, including inflammation, fibrosis, and cancer. While morphological change can be indicative of phenotypic changes, subcellular reorganization, or damage to the cytoskeleton, several subtle changes can only be observed using super-resolution imaging

and texture analysis. The incorporation of texture analysis in current research has been a boon to the medical imaging field. Texture analysis can be applied to analyze changes in the structure and organization of different organs as a result of pathology at several disease stages and to monitor the success of treatment over the lifetime of the patient.

Many commercial and open-source packages exist to segment objects from images, many of which include modules, small clusters of algorithms that can be applied to quantify images in step-by-step pipelines. Specific software made available independently or in conjunction with an imaging system include Imaris from BitPlane, Velocity from Improvision, Matlab Image Processing toolbox from MathWorks, ImageJ by Scion Corporation, Aperio ImageScope by Leica, CellProfiler developed by the Broad Institute, Harmony by Perkin Elmer, and Python and R programming languages, among other more specialized programs. To date, many applications of image analysis have used supervised analysis, an analysis where the user sets any number of parameters that drive image segmentation. Alternatively, several new programs and coding platforms are capable of unsupervised analysis, an analysis where the computer iteratively estimates differences between images to perform successful segmentation. For this research, supervised segmentation was utilized with user inputs that were focused on specific features of interest pertaining to biomarkers of myofibroblasts and fibrosis. Supervised segmentation has proved highly useful in evaluating medical samples and diagnosing pathology, measuring morphological features associated with organs, cells, and organelles, and monitoring the effects of treatment on patient health, cellular viability, and organelle function with the potential to identify spatiotemporal trends. With so many platforms and so much potential data, we must utilize these tools to their greatest capacity to push the limits of the fields of pathology, toxicology, and pharmacology forward.

Image segmentation of background thresholded images requires a robust confocal image for the correct segmentation of biologically-relevant objects. For this thesis research, images were generated from confocal single plane or multi-plane Z-stack images. Z-stacks refer to the capturing of image data at various levels within and throughout an object at steps of consistent depth. Z-stacks can be processed in multiple ways, including flattening of the Z-stack into a mean, max, or minimum projection or as a 3D object. Z-stack flattening minimizes computational costs by taking multiple images and turning them into one image. Flattened images evaluate all pixel values at the same location in each image and take the average, max, or minimum pixel value and translate that value to a new pixelated image of the same size. This new image now represents the mean, max, or minimum pixel value at every location that can be compared across experimental conditions. For reproducibility, only images generated from Z-stacks of the same height, image size, and processing type should be compared.

A primary step in image segmentation is thresholding, a method that requires a binary image that can be analyzed as a set of background and foreground pixels. Several types of image thresholding can be applied to microscopy images to determine which pixels are classified as background or foreground. Types of image thresholding include the maximum entropy method, balanced histogram thresholding, Otsu's method, and k-means clustering, among others. Maximum entropy in an image refers to the measure of disorder within an image, suggesting that if a certain pattern is real, the pattern is likely to be repeated within an image. When a pattern is repeated, the algorithm assumes the pattern to be real, normalizing the pixel values across the image to associate the identified patterns as foreground. Balanced histogram thresholding generates a histogram of pixel values within an image to identify the skew. The balanced histogram thresholding algorithm then balances the histogram to remove the skew and identify a value at which the pixel values diverge most effec-

tively into two classes. The divergent value is set as a threshold at which the pixels are categorized as foreground and background. The balanced histogram thresholding algorithm is similar to Otsu’s method, both of which work best at identifying background and foreground regions only, with intermediate and less robust signals being poorly characterized. K-means clustering is an unsupervised algorithm that can segment foreground from background pixels at several levels depending on the shape and scale of the pixel value distribution. K-means thresholding is ideal for more complex image sets with variable signal strength for a given output. While other, more specialized thresholding methods exist, these four algorithms are arguably the most common thresholding algorithms. For this thesis work, all thresholding was supervised and performed using absolute thresholding calculated from secondary-only antibody staining associated with non-specific binding. It was determined that the application of unsupervised thresholding algorithms performed poorly on small, punctate antibody signals. As a result, optimized image analysis methods were utilized on all images, even those with robust signals that did not need advanced filtration, to ensure the ability to compare between results.

In order to segment a thresholded image, images were put through a sliding parabola filter. A sliding parabola filter identifies pixels previously segmented as background and sets all background pixels to zero while normalizing the relative intensity of the foreground pixels. Sliding parabola filtration increases the robustness of the differences between background and foreground pixels and enhances the efficacy of object segmentation algorithms. Several techniques can be used to identify objects in images correctly. Objects in an image can be described as large, singular objects, like images of entire organs, or multiple small objects, like cells or sub-cellular compartments. While different segmentation algorithms exist and could be applied to train the computer to recognize objects intelligently, object segmentation was applied to foreground pixels with user supervision using an absolute threshold

as previously described. A common family of segmentation algorithms includes the watershed transformation, which evaluates an image as a topographic surface defined by the organization of the foreground pixels. Object “seeds” are identified by a high-intensity foreground pixel. The algorithm acts to identify “watershed lines,” defined as region boundaries, that identify an object moving away from the object “seed,” defining the edges of objects where pixel intensity decreases relative to the central “seed” pixel intensity. Additional algorithms, such as erosion and dilation algorithms, can be applied to shrink or expand the identified object area to increase or decrease, respectively, the margin around objects. These techniques are most accurate at maintaining correct morphological features when applied to round objects and is less often used when objects have minute details at the object edges.

Texture Analysis

Texture analysis is a class of mathematical procedures that measure spatial variation in images. Analyzing images using texture algorithms assigns a quantitative value to each image that can define features we might describe as smoothness, coarseness, and regularity. Fluorescence microscopy images consist of individual clusters of fluorescence values, pixels, from which relationships are extracted. These global measurements are considered whole descriptions of local intensity measurements that describe the relationships between independent pixels to one another within an image. Patterns characterize tonal variation in each image and can discriminate surface characteristics and patterns that are repeated, potentially characterizing images or objects based on the similarity of their organization. The texture analysis applied to the images evaluated by this thesis was performed using derivative images. Derivative images are generated by passing a convolution filter, 3x3 pixels, across the image to generate a less complex, derived image from which texture values are calculated (Figure 2.1). Each filtered region is normalized by intensity then averaged over the

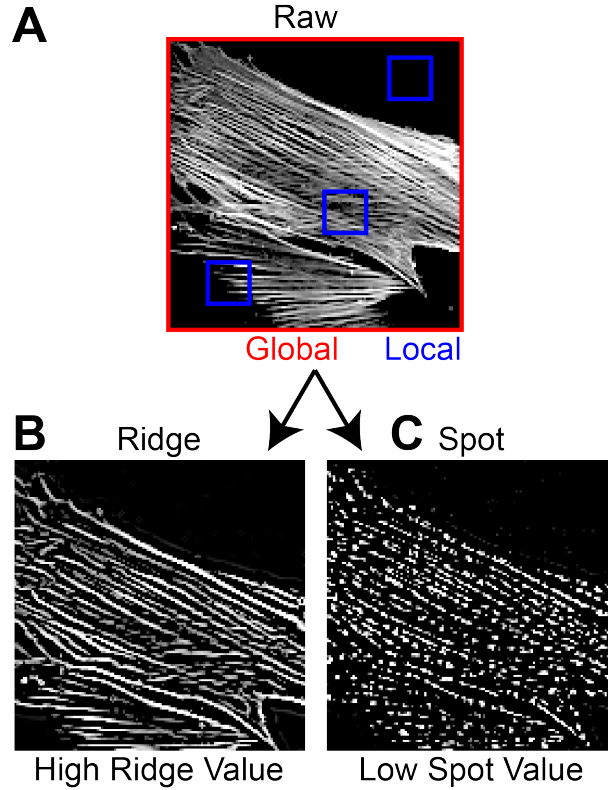


Figure 2.1: Texture Analysis

(A) The texture analysis utilized in this research is called a convolution filter, which uses local (blue box) and global (red box) convolution analysis. Convolution filtering requires a user to define the size (resolution) of a cluster (convolution) of pixels. (B) A high value suggests that the expected pattern or frequency of spatial interaction between high- and low-intensity pixels in a given region matches an expected spot, edge, or other texture types as pre-defined by Harmony software. Alternatively, (C) a low value is assigned when a texture algorithm poorly fits an image pattern.

segmented object to achieve a final object value. Harmony software by Perkin Elmer comes with eight intrinsic SER (spot, edge, ridge) Gaussian filters (Figure 2.2). Other types of texture features include Haralick and Gabor features. Haralick features were developed in the 1970s and describe general pixel relationships in an image by correlation, contrast, homogeneity, and sum variance. Haralick features do not require normalization by intensity. Gabor features are commonly used to identify scripts in documents because Gabor features are efficient at detecting patterns and directionality within images. Two parameters of Gabor features perform pattern detection by

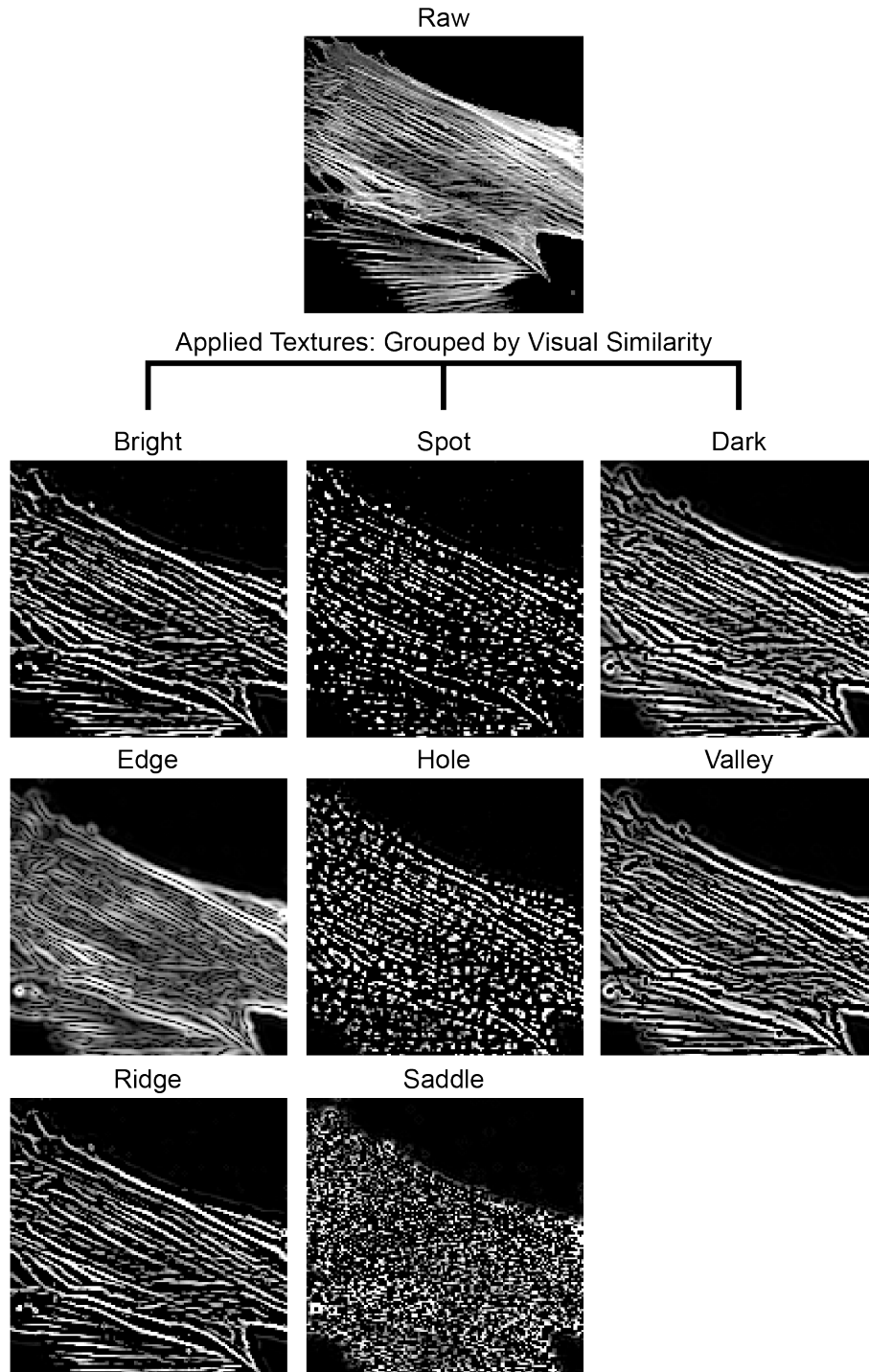


Figure 2.2: Representative Texture Algorithms

measuring the characteristic number of angles and are normalized in a similar way to SER features by intensity.

All in all, the application of advanced image segmentation techniques aid in the evaluation of physical and molecular changes associated with fibroblast to myofibroblast differentiation. Image segmentation is capable of identifying large, small, bright, dim, elongated, or punctate signals, allowing for the evaluation of several different types of biomarkers important for predictive fibrotic potential. Additionally, 2D in vitro models using immunofluorescence imaging techniques allow for the quantification of population-level and single-cell changes in different biomarkers in live or fixed samples. Therefore, evaluating subtle changes in population heterogeneity, like those previously observed in fibroblast populations, and spatiotemporal changes in fibroblast and myofibroblast phenotype become possible. Finally, the confirmation of small shifts in population phenotype observed as a result of non-chemical, particle exposures by molecular assays is difficult but becomes possible using multivariate analysis.

2.3 Materials and Methods

2.3.1 Cell Culture

IMR-90 (ATCC CCL-186) human, lung fibroblasts were cultured in cell culture treated flasks in Eagle’s Minimum Essential Medium (EMEM) (ATCC 30-2003) with 15% fetal bovine serum (Atlanta Biologicals S12495H) and 1% Penicillin-Streptomycin (Thermo Fisher 15140122). Cell lines were cultured to below 85% confluence before splitting and utilized until passage 15. Cells were collected and centrifuged for 5 minutes at 1000 rpm at 4°C. 2D experiments on 5 $\mu\text{g}/\text{cm}^2$ collagen-coated plastic were plated with 3,125 cells per cm^2 in 200 μL per well in 96-well plates (Greiner 655956). Cells were seeded onto 5 $\mu\text{g}/\text{cm}^2$ collagen-coated polyacrylamide gels at 5,700 cells per cm^2 in 1 mL per well in a 24-well plate (Eppendorf 0030741021). All plates were placed in an incubator with 5% CO_2 at 37°C overnight to promote cell adherence before treatment with nanomaterials or 5 ng/mL TGF- β 1

(R&D Systems 240-B-010). Cells were fixed in 10% neutral buffered formalin at 48 hours for p-Smad2/3 endpoints and 72 hours for α -SMA endpoints.

TGF- β 1 and Nanomaterial Dilution

5 μ g of lyophilized recombinant TGF- β 1 (R&D Systems 240-B) is suspended to 20 μ g/mL in 4 mM HCl and 1 mg/mL BSA. All TGF- β 1 controls are diluted from 20 μ g/mL to 5 ng/mL in incomplete media (EMEM, 2.5% FBS, and 1% P/S) unless otherwise stated.

2.3.2 Preparation of Nanomaterial Suspensions

Carbon black (M120), consisting of isometric elemental carbon nanoparticles, was purchased from Cabot Corp. (Boston, MA) and, as previously characterized, has a primary diameter of 75 nm and a total surface area of 35 m²/g. Multi-walled carbon nanotubes (MWCNT-7) from Mitsui and Co. Ltd. (Tsukuba Ibaraki, Japan) were generously provided by Dr. Gunter Oberdörster (the University of Rochester, Rochester, NY). The 550 nm and 5 μ m graphene flakes were purchased from Cabot and Graphene Supermarket, respectively. The physiochemical descriptions of these materials are summarized in Table 2.2. To ensure sterility and maintenance of the endotoxin-free environment, all material stock suspensions were prepared using sterile methods under a Class IIB biological safety hood with external exhaust. Nanomaterial stocks were suspended in sterile, endotoxin-free, 10% dipalmitoylphosphatidylcholine (DPPC, Avanti Polar Lipids 850355P) and 3% BSA (Sigma-Aldrich A9576) solutions. Stocks were water bath sonicated (Branson Ultrasonic Corporation, Danbury, CT) for 5 minutes and brought to 1000 μ g/mL concentrations in 1% bovine serum albumin-containing (BSA) (Sigma-Aldrich) media and then water bath sonicated for an additional 45 minutes. Stocks were kept at 4°C and sonicated again prior to dilution to working stock concentrations in complete EMEM cell culture media.

This dispersion technique produced uniform suspensions of individual particles and flakes (carbon black and graphenes) or individual fibers and small agglomerates (carbon nanotubes) as described previously [102]. Endotoxin levels in the stock particle suspensions were determined using the gel-clot assay and were less than 0.31 EU/mL (Associates of Cape Cod, Inc.). 1000 $\mu\text{g/mL}$ stocks of nanomaterials are diluted in incomplete media (EMEM, 2.5% FBS, and 1% P/S) to 3.6 $\mu\text{g/mL}$ concentrations, equal to a seeded concentration of 0.6 $\mu\text{m/cm}^2$ on a T-150 flask. Concentrations of carbon nanotube exposure corrected for lifetime exposures were calculated based on the risk-based low-dose extrapolation for non-cancer endpoints. Inhaled workplace concentrations were corrected to equate to the “human-equivalent lung response after 45-year exposure (respirable mass, 8-hour, time-weighted average concentration, 40-hour work week, 50 weeks per year) and were defined as being “associated with 0.1% excess risk at 0.0051 $\mu\text{g/m}^3$.” Back calculation to 1.2 $\mu\text{g/cm}^2$ per year concentrations were used as a starting concentration for preliminary viability assays on carbon black (negative particle controls), carbon nanotube (known toxic fibrous nanotubes), and untreated conditions. 2.4 and 1.2 $\mu\text{g/cm}^2$ carbon nanotube exposures resulted in toxicity, while fibroblasts exposed to 0.6 $\mu\text{g/cm}^2$ carbon nanotubes exhibited >90% viability. Values are corrected for clearance kinetics, ventilation rate, and alveolar deposition fraction [100, 101].

RNA isolation, PCR arrays, and qRT-PCR assays

To collect RNA, samples were rinsed in PBS twice and lysed with TRI-Reagent (MRC, Cincinnati, OH). Total RNA was then isolated as described previously [46]. For analysis of gene expression using Human Fibrosis RT2 Profiler PCR Arrays (Qiagen PAHS-120Z), DNase treated RNAs were cleaned through RNeasy® MinElute™ columns, then converted to cDNAs and used for analysis according to vander specifications (Qiagen, Germantown, MD). Resulting data were uploaded to the Qiagen web portal

(www.qiagen.com), normalized to averaged reference transcripts with differences in CT value less than one across all sample groups, and evaluated using $\Delta\Delta\text{CT}$. Genes with statistically significant gene expression changes ($p\text{-value} < 0.05$) and/or changes greater than 2-fold when compared to untreated samples and displayed as heat maps or volcano plots.

Proteomics: Sample Collection

IMR-90 cell samples were collected for proteomics analysis to explore changes in protein driven by 5 ng/mL TGF- β 1 and 0.6 $\mu\text{g}/\text{cm}^2$ nanomaterial exposure as compared to untreated cell controls. IMR-90 cells were seeded in T-150 flasks to 30% cell confluence overnight to adhere in complete media (EMEM, 10% FBS, and 1% P/S). Complete media is aspirated from adherent IMR-90 cells, and cells are washed with 1x PBS. Plain incomplete media (EMEM, 2.5% FBS, and 1% P/S) media, incomplete media with 5 ng/mL TGF- β 1, or incomplete media with 3.6 $\mu\text{g}/\text{mL}$ nanomaterial is added to flasks. The treated flasks incubate for 72 hours at 37°C with 5% CO_2 . The collection of protein from IMR-90 cells at 72hour is performed on ice. Flasks are rinsed with 1x PBS and treated with 0.25% Trypsin-EDTA for 3-5 minutes until cells are non-adherent. Cell suspensions are moved into 15 mL conicals on ice and centrifuged at 1000 rpm and 4°C for 5 min. The supernatant is aspirated from cell pellets, and pellets are resuspended to wash in 1x PBS. Cell suspensions are moved into 1.5 mL Eppendorf tubes on ice and centrifuged again at 1000 rpm and 4°C for 5 min. The supernatant is aspirated from the washed cell pellet, and the pellet is placed at -80°C until processing to collect protein. The CCRD proteomic core facility performed sample processing to collect intracellular protein and analysis by mass spectrometry. Provided samples included five experimental replicates of over 10 million cells per sample achieved through harvesting five T-150 flasks per sample.

Proteomics: Data Processing and Statistical Analysis

Raw data from mass spectrometry was analyzed in-house by the CCRD proteomic core facility to yield normalized raw and average abundance values for under 16000 peptides belonging to approximately 4000 proteins.

Proteomics: Volcano Plots

Normalized abundance values from the CCRD proteomic core facility are processed using R programming language employing a Bayesian moderated T-statistic to test each individual peptide against untreated sample peptides and then corrected for multiple comparisons by F-statistic settings specifying a false-discovery rate (FDR) of 0.001. The output t-statistic and associated q-statistic (adjusted p-value) are matched to an average peptide abundance across the proteome. A list of the top 50 genes for each sample comparison to untreated samples was generated and illustrated as a Venn diagram. To process data for volcano plots, peptide abundance was summed for each protein and divided by the matching untreated sample to calculate a fold change. Further analysis done in GraphPad Prism 8 utilizes multiple t-tests to calculate the significance of each protein to all other proteins in the dataset to calculate a q-statistic (adjusted p-value) for every protein. Assumptions chosen for multiple t-tests include similar scatter across conditions and experiments and multiple comparison p-value corrections using the Holm-Sidak method with an alpha of 0.05. The two-stage step-up method of Benjamini, Krieger, and Yekutieli with a false discovery rate of 0.01 (1 in 100 FDR per 4000 proteins, as opposed to the previously used 1 in 1000 FDR for 16000 peptides) yields almost all of the same top 50 proteins found by the Holm-Sidak method, leading to the use of the Holm-Sidak method. The multiple t-test output provides the q-statistic that was matched to the protein log(fold change) to generate volcano plots (typically illustrated as log(fold change) by

p-value). Calculated fold change values are also displayed as a heatmap for all cell samples compared to untreated samples.

Proteomics: Ingenuity Pathway Analysis (IPA)

IPA analysis takes processed fold change values with corresponding gene ontology identifiers to calculate a list of the top five “Cellular and Molecular Pathways.” While analysis pipelines for IPA are proprietary, company descriptions cite the use of current literature and proteomics analysis pipelines to relate protein signatures to known signaling pathways to identify enriched molecular pathways.

2.3.3 Polyacrylamide Hydrogel Substrates

Polyacrylamide (PA) hydrogel substrates gels were prepared in 24-well plates (Eppendorf 0030741021) following the protocol described by Syed et al. 2015 [99]. 2 kPa PA gels were made by adding 2% bis-acrylamide solution (BioRad 1610142) and 40% acrylamide solution (BioRad 1610140) to 10% w/v ammonium persulfate (Thermo Fisher 17874) and TEMED (Sigma T7024) according to the volumes provided in Supplemental Table 2. Complete PA solutions were vortexed, and 16 μ L was pipetted into each well and covered with an 8 mm hydrophobic coverslip. This volume was confirmed to produce gels at least 100 μ m in height to minimize mechanical interactions between cells and the rigid glass substrate. The gels were further modified for sulfo-SANPAH crosslinking to collagen I to a final concentration of (5 μ g/cm² rat-tail, Sigma C3867) following the protocol described in Syed et al. 2015 [99]. Collagen-coated plates were kept at 4°C in 1x PBS overnight. The plate was rinsed with 1x PBS twice and equilibrated in complete EMEM cell culture media before adding cells. Table S2.4 describes the formulations for tunable PA gels.

2.3.4 Cell Viability

Fibroblasts were cultured and exposed to exogenous TGF- β 1 or nanomaterials according to protocols stated above. The media was aspirated from control wells and replaced with 100 μ L or 500 μ L of 70% ethanol, in 96-well or 24-well plates, respectively, 1 hour prior to staining to produce a dying cell population. Cells were stained in 100 μ L or 500 μ L of red/green stain working solutions, in 96-well or 24-well plates, respectively, according to LIVE/DEADTM Cell Imaging Kit (Thermo Fisher R37601) protocols and 1:1000 Hoechst 33342 (Thermo Fisher 62249). Fibroblasts on stiff, collagen-coated plastic were imaged on an Opera Phenix (Perkin Elmer) spinning disk confocal microscope. Calcein AM stain, indicating live cells, was imaged using a 488 nm excitation laser with a 50 ms exposure time and 5% laser power. Hoechst stain, indicating cell nuclei, was imaged using a 405 nm excitation laser with a 100 ms exposure time and 25% laser power. Ethidium Homodimer-1 stain, indicating dying cell nuclei, was imaged using a 568 nm excitation laser with a 100 ms exposure time and 10% laser power. Fibroblasts on 2 kPa polyacrylamide were imaged on an Opera Phenix (Perkin Elmer) spinning disk confocal microscope. Calcein AM stain, indicating live cells, was imaged using a 488 nm excitation laser with a 100 ms exposure time and 20% laser power. Hoechst stain, indicating cell nuclei, was imaged using a 405 nm excitation laser with a 200 ms exposure time and 15% laser power. Ethidium Homodimer-1 stain, indicating dying cell nuclei, was imaged using a 568 nm excitation laser with a 200 ms exposure time and 15% laser power. Both viability samples were imaged using a 10x Air objective.

2.3.5 Nanomaterial Uptake and Lysosomal Integrity

Cells were cultured on rigid substrates and exposed to exogenous TGF- β 1 or nanomaterials according to protocols stated above. After 24 hours, control wells were treated with 10 μ g/mL ciprofloxacin HCl (GenHunter Corporation Q902) 5 minutes prior to

cathepsin-B staining to induce cathepsin-B leakage. After 5 minutes, all cell populations were stained according to Magic Red cathepsin-B kit (Bio-Rad ICT937) protocols. Separate treated 24-hour cell populations were stained with 17 nM lyso-tracker (Thermo Fisher L7528) solutions. (Thermo Fisher protocols recommend 50-75 nM staining concentrations, but 50 nM exposure resulted in cell death between 60 and 90 minutes after staining that was not observed after cathepsin-B staining or live/dead staining. To minimize artifacts of apoptosis and because imaging took approximately 1 hour to complete, concentrations were optimized to be as low as possible.) Samples were imaged on an Olympus IX81 confocal microscope. Images were normalized by expanding each image's intensity spectra to the highest and lowest intensity spectra value within each image set using batch normalization. Rigid substrate fluorescent lysotracker and cathepsin-B labels were imaged using a 561 nm excitation laser with a 63x water objective, a 100 ms exposure time, and 20% laser power.

2.3.6 Fluorescence Imaging

To evaluate the presence of p-Smad2/3 complex, α -SMA, and collagen 1 α 1 protein endpoints, cells were prepared, cultured, and exposed to exogenous TGF- β 1 or nano-materials according to protocols stated above. Cells were fixed in 10% neutral buffered formalin and incubated at 4°C overnight before processing. Fixed samples were permeabilized in 0.25% Triton X-100 and blocked with blocking buffer for 1-hour in 5% BSA, 5% goat serum (Sigma G9023) or donkey serum (Sigma D9663), and 0.3 M glycine in 1x PBS. Blocked samples were stained overnight at 4°C with rabbit polyclonal p-Smad2/3 (Cell Signaling 8685, 1:100) and mouse monoclonal alpha-smooth muscle actin (Cell Signaling 19245, 1:250) antibodies. For collagen 1 α 1, blocked samples were stained overnight at 4°C with 5 μ g/mL sheep collagen 1 primary antibody (R&D Systems AF6220, 1:40) solution diluted from a stock concentration of 200 μ g/mL. Primary antibodies were fluorescently-labeled with goat anti-rabbit

(AF488, Cell Signaling 4412), goat anti-mouse (AF647, Cell Signaling 4410), or donkey anti-sheep fluorescein-conjugated antibody (R&D Systems F0125) at a final dilution of 1:1000. Control wells received 1:1000 secondary antibody solutions. Samples were counterstained with 1:1000 Hoechst 33342 (Thermo Fisher 62249) and 1:100 rhodamine-phalloidin (Thermo Fisher R415) and imaged on an Opera Phenix (Perkin Elmer) spinning disk confocal microscope. The average nuclear p-Smad2/3 and cellular α -SMA content was analyzed using Perkin Elmer Harmony software. Rigid substrates were imaged using 40x water objectives. Rigid substrate fluorescent p-Smad2/3 labels were imaged using a 488 nm excitation laser with a 400 ms exposure time and 20% laser power. Rigid substrate fluorescent α -SMA labels were imaged using a 647 nm excitation laser with a 70 ms exposure time and 10% laser power. Soft substrates were imaged using 20x water objectives. Soft substrate fluorescent p-Smad2/3 labels were imaged using a 488 nm excitation laser with a 100 ms exposure time and 20% laser power. Soft substrate fluorescent α -SMA labels were imaged using a 647 nm excitation laser with a 160 ms exposure time and 20% laser power. Rigid substrate fluorescent collagen 1 α 1 labels were imaged using a 488 nm excitation laser with a 840 ms exposure time and 35% laser power.

2.3.7 Image Normalization and Quality Control

Rigid substrate datasets were collected in one image plane. Soft substrate datasets were collected at the height of 100-160 μ m from the bottom of the plate to at 1.5 μ m intervals. All datasets were treated as max projections with all background regions, regions without segmented objects, set to a value of zero. Individual imaged regions across each plate well were stitched together (5 μ m overlap between frames across the bottom of each well using a 20x water objective) and treated as a singular global image (Figure S2.31A). Due to the incline of manually produced polyacrylamide gels, a subset of the cell population comes into focus when maximum

projections are generated from each Z-stack. As a result, normalization by cell count or clustering was inaccurate and not performed for rigid or soft substrate datasets. Experiments were performed with three "technical replicates" per plate and three total experiments (three different plates, different IMR-90 cell populations) performed for each substrate. IMR-90 cells were utilized below passage 15 and monitored for early senescence indicated by a doubling time greater than 24 hours.

2.3.8 Nuclear and Actin Segmentation

504 nm and 568 nm excitation spectra were utilized for nuclear and actin labels, respectively. Rigid and soft substrate max projection nuclear and actin channel images were filtered using a sliding parabola filter optimized at a curvature of 90 and 30, respectively. This was optimized by eye for nuclear and actin channels with three goals, to minimize loss of intrinsic intensity variability within nuclei, to prevent loss of individual actin fiber resolution in global images, and to minimize background fluorescence. This greatly reduced background signal observed within PA substrates even after washing, but only marginally impacted rigid substrate images, as expected. Nuclei were selected as being brighter than 80% of image pixels, with an area greater than $30 \mu\text{m}^2$ to prevent false puncta detection and had a contrast greater than 10% of the image. Nuclei were further filtered by location and size. If nuclei were touching the edge of a global image, were less than $150 \mu\text{m}^2$ or greater than $1100 \mu\text{m}^2$, or greater than 0.6 roundness (>0.6 roundness often correlated with a higher overall intensity suggesting apoptosis or mitosis), they were excluded from the dataset. Nuclear area and roundness were included as part of the feature set for each cell. Actin was not thresholded as the signal was so robust that sliding parabola filters set background signal to zero.

2.3.9 Biomarker Segmentation

Around each nucleus, a perinuclear region was defined to include the nucleus with a 20 μm radius from the nuclear edge. Images were collected using Alexa Fluor 488 secondary antibody labels for p-Smad2/3, Alexa Fluor 647 secondary antibody labels for α -SMA. p-Smad2/3 and α -SMA biomarkers were quantified within this perinuclear region using the specified channels. Each plate was prepared with three Alexa Fluor secondary antibody-only treated wells for each biomarker label. The highest intensity value in “secondary only” wells was averaged to calculate the background-level intensity. To identify p-Smad2/3 and α -SMA regions, each well was thresholded by the lowest intensity being greater than or equal to the calculated background level intensity as background subtraction. As puncta for p-Smad2/3 complexes can be small, any signal greater than a single pixel (1 px^2) within nuclear regions was considered “real,” while any signal greater than 10 px^2 within the perinuclear regions was considered “real” for α -SMA (SFig.XD). Only the nuclear p-Smad2/3 complex was considered to limit biomarker detection to active transcription factor complex. α -SMA biomarker size was optimized to account for what could be a robustly formed α -SMA cytoskeleton or partial α -SMA cytoskeleton development and to exclude diffuse or punctate α -SMA signal. From the “real” biomarkers identified in each cell, the mean intensity, mean contrast, and total percent area of the biomarker were calculated and added to the feature set. Classification of cells as undergoing myofibroblast differentiation or as a myofibroblast was defined as $>20\%$ nuclear or perinuclear area. The whole-cell morphological analysis could not be performed without cell membrane markers. This goal was unattainable using any “cluster of differentiation” (CD glycoprotein) membrane protein, N-cadherin, CellTracker stain, focal adhesion, or cell-cell junction labels to produce a discrete cell edge for segmentation.

2.3.10 Texture Analysis

Texture analysis is a class of mathematical procedures that measure spatial variation in images. Fluorescence microscopy images consist of individual clusters of fluorescence values, pixels, from which relationships are extracted. The texture analysis utilized in this research is called a convolution filter, which uses local and global convolution analysis. Convolution filtering requires a user to define the size (resolution) of a cluster (convolution) of pixels. For example, a one-pixel resolution applies to each pixel and its corresponding eight adjacent pixels with a one-pixel step as the convolution moves across an image. In fluorescent images, each calculated convolution value was normalized to the intensity of each pixel. Texture features in this paper were collected using the aforementioned one-pixel resolution, the highest resolution possible, in an attempt to capture as much detail as possible. Thus, each local texture value is characterized by intensity and location properties, while the global object texture is the average of local texture values. As texture values were calculated within a defined segmented perinuclear region, whole image texture data was not statistically evaluated. A higher texture value notes the strength of a given texture type. Figure 6S contains representative images of all of the applied texture functions.

2.3.11 Single-cell Feature Set Analysis

Features were extracted from each experiment, given a plate designation, and merged into one dataset. Other categorical features in the dataset included phenotype, as defined as $>20\%$ of a biomarker region, and treatment condition. Before statistical evaluation, texture features were normalized to region intensity during image analysis and were rescaled between zero and one across each plate to control for intensity and individual plate deformities. Features were then analyzed to compare cell populations across treatments using multivariable regression, Factor Analysis of Mixed Data (FAMD) principle component analysis, t-Distributed Stochastic Neighbor Embedding

(t-SNE), and evaluated for significance by non-parametric ANOVA and feature correlation within R. The libraries and code used for this analysis are available and can be accessed using the above link.

2.3.12 Statistical Analysis

The assumptions to run a parametric ANOVA on these datasets were not met. To run a non-parametric ANOVA (pairwise Wilcoxon test with a Benjamini and Hochberg (BH) post-test) for single-cell feature profiles compared to untreated conditions, the feature dataset was compiled into one column with its matching feature description as a separate column and then across all features according to phenotype and exposure designations. Datasets were separated into two datasets by phenotypic designation into fibroblasts and intermediate myofibroblast (denoting active differentiation) or myofibroblast phenotypes. The ANOVA was then performed to compare all treatments to one another across phenotypes.

Model selection was performed in place of multivariable regression to identify the best fit of groups of variables that were best capable of predicting cell phenotype and treatment. The groups of features that explained the most variation between phenotypes (which features best predict fibroblast vs. myofibroblast classification) and treatment populations (which features best indicate treatment condition) were calculated and were visualized by adjusted p-value (data not shown).

All data are reported as mean and standard deviation (SD) unless otherwise stated. Viability data were analyzed using a one-way ANOVA with Dunnett's post-test (Graphpad Prism 6 software). Data for image analysis was collected using Perkin Elmer Harmony image analysis software with multivariate analysis performed in R statistical software. Descriptions of quality controls, filtering, and analysis details used in feature extraction are described above. Statistical difference between fibroblast and myofibroblast phenotypic profiles was calculated using a two-way ANOVA

with a Dunnett's post-test (Graphpad Prism 6 software). Statistical difference between treatment in separated fibroblast and myofibroblast phenotypic profiles was calculated using a pairwise Wilcoxon test with a Benjamini and Hochberg (BH) post-test (R programming language), also described in detail above. The statistical tests were chosen contingent on meeting the correct analytical assumptions required for each test and are further described above. All experiments were repeated a minimum of three times.

2.3.13 Hierarchical Clustering and FAMD Plotting

For all clustering and correlation analyses, feature data was scaled. Hierarchical clustering with an applied unsupervised K means was performed to evaluate the differences between treatments based on scaled Euclidean distance (overall distance between cells in each treatment). FAMD plots were generated and overlaid with a 95% confidence ellipse for both phenotype and treatment groups. (If more experiments were performed, 95% of all generated values are predicted to fall within the overlaid ellipse.)

2.4 Results and Discussion

The main objective of this research is to incorporate previously established biomarkers of fibroblast to myofibroblast differentiation into a screening tool to predict nanomaterial toxicity in the human lung. Five key features of the experimental design were considered to maximize screening tool utility. 1) Untreated controls were compared to TGF- β 1 exposure, a potent inducer of fibroblast to myofibroblast differentiation. 2) Primary IMR-90 fibroblasts were utilized as a model cell line because they are readily available, cost-effective, and un-transformed lung fibroblasts. 3) Particle controls were incorporated to detect differences between toxic and non-toxic particle

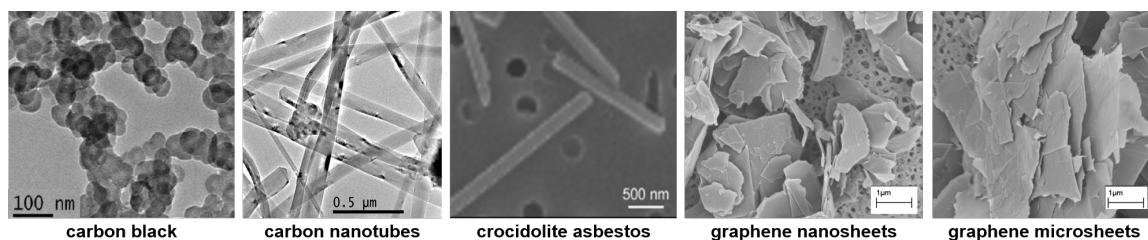


Figure 2.3: Representative Nanomaterial Images

Carbon black, carbon nanotube, asbestos, and graphene nano- and micro-sheet nanomaterials were imaged using high-resolution electron microscopy to observe particle shape and geometry.

exposures, including well-characterized carbon black, Mitsui carbon nanotubes, and crocidolite asbestos. 4) We incorporated a soft substrate to mimic the healthy human lung, and all substrates were coated in 5 $\mu\text{g}/\text{mL}$ collagen type 1. 5) And the model system was developed in tandem with an image and statistical analysis pipeline that is capable of analyzing a wide array of cell phenotypes beyond this in vitro screening tool.

2.4.1 Assembling A Nanomaterial Panel

The use of in vitro and in vivo models in toxicological research often requires the use of high concentrations of a toxicant to measure a response. Therefore, another primary goal of our proposed in vitro screening tool is to predict nanomaterial toxicity at concentrations that are within range of potential human exposure in the workplace as reviewed by the National Institute for Occupational Safety and Health (NIOSH). Nanomaterials of variable size and shape were chosen as part of a panel of well-characterized or emerging nanomaterial contaminants (“emerging” with respect to increasing industrial production). Representative images of the chosen nanomaterial panel highlight similarities in particle geometry between two fibrous nanomaterials (carbon nanotubes and crocidolite asbestos) and two graphene sheets (nano- and micro-sheets) (Figure 2.3).

Sample and Origin	Length (μm)	Diameter (nm)	Fe content (wt. %)	Surface area (m ² /g)	Hydrodynamic size (μm)	Stiffness (kPa)
Carbon black: M120 Cabot Corp.	N/A	75	ND	35	0.273	N/A
Carbon Nanotubes: MWCNT-7 Mitsui & Co.	11.7 ± 3.6	80 ± 18	< 0.2	35	1.379	3.6-63 x 10 ⁶
Crocidolite asbestos UICC	2.8 ± 2.6	116 ± 112	22.2	9.1	ND	1.1-4.4 GPa
Sample	Lateral dimension (μm)	Number of Layers	Thickness (nm)	Surface area (m ² /g)	Stiffness (monolayer) (kPa)	
G550: Graphene Cabot Corp.	0.550	25	8.5	25	ND	
G5: Graphene Graphene Supermarket	5	20	7	115	130 x 10 ⁶ , modeled: 118-121 x 10 ⁶	

Table 2.1: Nanomaterial Panel of Physicochemical Characteristics
Carbon black, carbon nanotube, asbestos, and graphene nano- and micro-sheet nano-materials were characterized in-house to confirm size, shape, and other key characteristics associated with each material.

Table 2.1 describes the characterization of each material. Inhaled carbon black particles are confirmed to be non-toxic in human case studies and are used as negative particle controls in our experiments. Additionally, the mechanisms of toxicity of inhaled crocidolite asbestos are well-established through human case studies. Fibrous asbestos is one positive particle control. However, crocidolite asbestos has a unique chemical make-up as a mined, fibrous silicate mineral. Mitsui multi-wall carbon nanotubes are also known to be fibrogenic and potentially carcinogenic in in vivo and in vitro models but are exclusively comprised of carbon. Mitsui multi-wall carbon nanotubes are used as a secondary positive control particle, sharing similar surface area characteristics with non-toxic carbon black. Previous in vivo research confirms that asbestos and carbon nanotube exposure can cause fibroblast to myofibroblast

differentiation, either directly or indirectly, which we expect to observe in our model. Finally, the incorporation of more novel graphene sheets introduces another geometry. While graphene research in in vivo and in vitro toxicity models is extensive and growing, the current literature reaches conflicting conclusions regarding the toxicity of pristine carbon graphene sheets of different sizes and concentrations. The graphene nano-sheets included in our panel are twice the size of carbon black particles with a similar surface area, and we hypothesized that graphene nano-sheets would not induce toxicity or fibroblast to myofibroblast differentiation. Finally, the graphene micro-sheets have a lateral dimension between the length of the asbestos and carbon nanotube fibers. While some in vivo models found that large graphene micro-sheets were toxic after inhalation, the two graphenes serve as test nanomaterials to evaluate the efficacy of our screening tool and analysis platform. We hypothesized that if graphene micro-sheets did not drive fibroblast to myofibroblast differentiation, image and statistical analysis would find the two graphene exposures to be more similar to one another than to particle or fiber controls. Finally, we considered two key characteristics that would impact the evaluation of biopersistent nanomaterials. 1) Every cell will observe a different concentration based on nanomaterial deposition or uptake, and 2) cell-nanomaterial interactions will produce mechanical stimuli in direct competition to the rigid, plastic in vitro cell culture substrate. We referenced the current literature to assemble the relative stiffness of each nanomaterial and considered the known stiffness of a healthy human lung (2-8 kPa) and polystyrene cell culture plates ($>10^6$ kPa). We hypothesized that larger fibers and graphene micro-sheets, with stiffnesses higher than a polystyrene substrate ($>10^7$ kPa), would increase fibroblast to myofibroblast differentiation relative to graphene nano-sheets.

2.4.2 Profiling Fibroblast Phenotypes After TGF- β 1 and Nanomaterial Exposure

Optimizing TGF- β 1 and Nanomaterial Exposure Concentrations

Initial experiments evaluate positive and negative control populations of IMR-90 primary lung fibroblasts to test for the induction of fibroblast to myofibroblast differentiation through the canonical TGF- β signaling pathway, which we activate through recombinant TGF- β 1 exposure. Optimal concentrations between 2.5 $\mu\text{g/mL}$ and 5 $\mu\text{g/mL}$ of TGF- β 1 induce differentiation in most of the fibroblast population after 72 hours (Figure S2.21) and do not cause the cell death observed by eye at 10 $\mu\text{g/mL}$ (Figure S2.22). All subsequent positive control fibroblast populations are exposed to 5 $\mu\text{g/mL}$ TGF- β 1.

After a 72-hour exposure (Figure S2.23), we confirmed that fibrous carbon nanotube and asbestos nanomaterials are most toxic to the fibroblasts (Figure S2.24). Viability assays show that exposure to 0.6 $\mu\text{g/cm}^2$ of either carbon nanotubes or asbestos causes approximately 20% cell death (p-value<0.05). Additionally, negative control carbon black causes no cytotoxicity at any concentration (Figure S2.24). All subsequent fibroblast populations are exposed to 0.6 $\mu\text{g/cm}^2$ of each nanomaterial, and fibroblast viability is reconfirmed for in vitro substrate.

Proteomics Analysis Identifies A Canonical Myofibroblast Marker After A 72 hour Carbon Nanotube Exposure

Proteomics analysis explored changes to the fibroblast proteome after nanomaterial exposure and was intended to inform experiments by identifying potential targets for antibody labeling and immunofluorescence imaging. Figure S2.25 illustrates the plating and exposure paradigm for proteomics sample collection after 72-hour exposures to carbon black, carbon nanotubes, and asbestos particle controls. Protein

was collected from treated fibroblast samples and analyzed by the CCRD proteomic core facility to yield normalized raw and average abundance values for under 16000 peptides belonging to approximately 4000 proteins (Figure S2.26). Median normalization normalizes the data before the calculation of peptide fold change from untreated samples (Figure 2.26A). When normalized peptide profiles are graphed in decreasing median order, a trend emerged that was subsequently illustrated more clearly by hierarchical clustering (Figure 2.26B). This suggests that TGF- β 1 samples are more similar to untreated profiles than particle-exposed profiles. In order to better observe changes at the protein level, peptides from each protein were summed, averaged across experimental replicates, and fold changes at the protein level are calculated. After multiple t-test analysis, log(fold changes) are plotted against adjusted p-values to generate heatmap and volcano plots. An increasing number of significantly down-regulated proteins are observed after 72-hour carbon black, carbon nanotube, and asbestos exposures. Adjusted p-values are plotted as a heatmap to illustrate changes in each sample's proteome (Figure S2.27). Volcano plots illustrate significantly different proteins at fold changes greater than two-fold up- or down-regulated. Most significant changes are not shared between conditions, and most proteins in the proteome are not significantly changed, which is further validated by volcano plots identifying significant peptide changes at fold changes >2 (Figure S2.28A) after TGF- β 1 exposure. Several more significantly down-regulated peptides were identified after carbon black (Figure S2.28B), carbon nanotube (Figure S2.28C), and asbestos (Figure S2.28C) exposures. Furthermore, to evaluate similarities between the top 50 differentially abundant peptides after each exposure, 50 peptides are pulled from each sample set and illustrated using a Venn diagram (Figure S2.29). After housekeeping peptides are removed, the approximately 30 remaining peptides are associated with cytoskeletal, calcium-regulation, and ECM pathways. These three pathways are critical pathways in mesenchymal cell regulation and are known to be

changed during and after fibroblast to myofibroblast differentiation. Additionally, the 72-hour carbon nanotube exposure resulted in an up-regulation of smooth muscle cell-associated peptides (ACTA2), also known as α -smooth muscle actin (α -SMA). Analysis of the proteome by Ingenuity Pathway Analysis software identified several up- and down-regulated proteins in each sample set that corresponded to significant pathway enrichment. Protein ontology identified several hundred proteins in each sample set associated with similar molecular pathways. Nanomaterial-exposed samples had significant changes to RNA post-transcriptional modification pathways and gene expression pathways, suggesting a shift in gene expression not observed after TGF- β 1 exposure. All exposures impacted proteins associated with cellular movement and protein synthesis, with TGF- β 1 exposure resulting in significant changes to cellular assembly and organization, all of which are associated with changes to the cytoskeleton (Figure S2.30). In order to further evaluate these changes, subsequent experiments incorporate two biomarkers of the canonical TGF- β pathway, transcription factor complex p-Smad2/3 (an intermediate marker of fibroblast to myofibroblast differentiation) and α -SMA (endpoint myofibroblast marker).

Fibroblasts Exhibit Minimal Particle Uptake Over Time

IMR-90 fibroblasts were cultured in 96-well plates (Figure S2.32A) for 24-, 48-, or 72 hours depending on the experimental endpoints tested (Figure S2.32B). The introduction of a collagen type 1 coating increased fibroblast viability after 72 hours (Figure S2.33) with and without exposure to particles, with decreases in viability associated with asbestos and graphene micro-sheets ($p < 0.05$) (Figure S2.34). We hypothesized that viability was not impacted due to minimal uptake of particles, which we confirm using lysosome and cathepsin-B stains. Under brightfield settings, nanomaterials are observed on top of cells and remain in focus just above cell bodies at 72-hour timepoints. In an attempt to observe nanomaterial uptake into cells, fibrob-

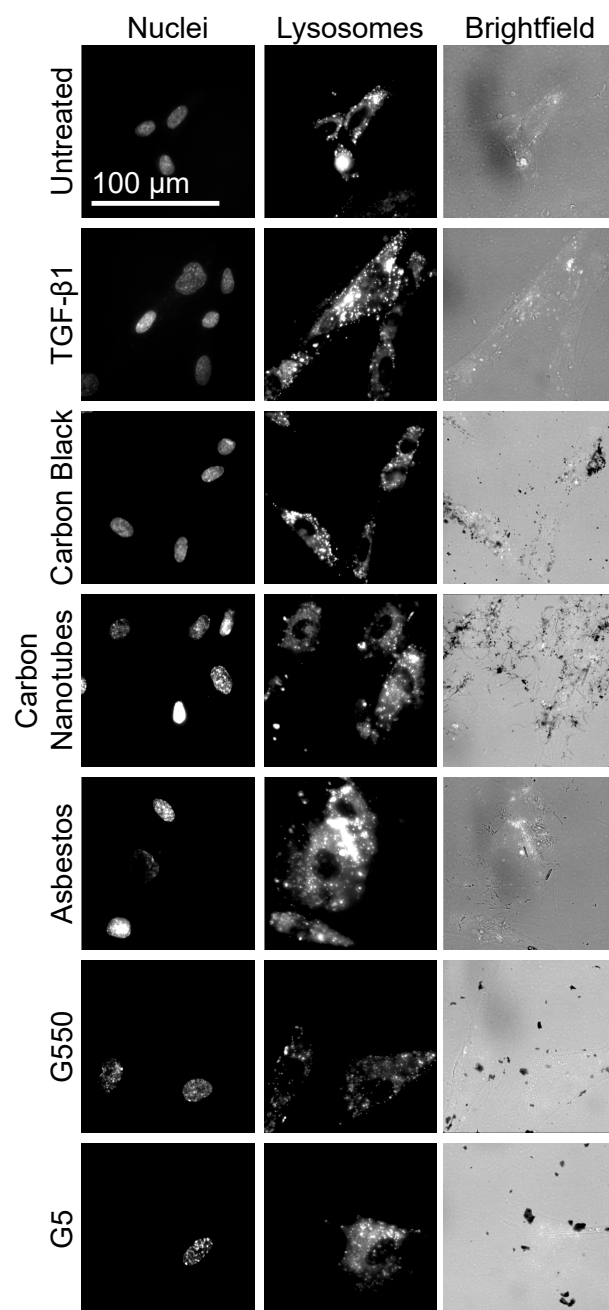


Figure 2.4: Lysosome-Particle Colocalization Suggests Minimal Particle Uptake
 IMR-90 fibroblasts were cultured for 24 hours under all experimental conditions and stained with LysoTracker lysosome stain. Lysosome stain and particle do not colocalize, and punctate lysosomes are visible in every condition. Scale bar = 100 μm .

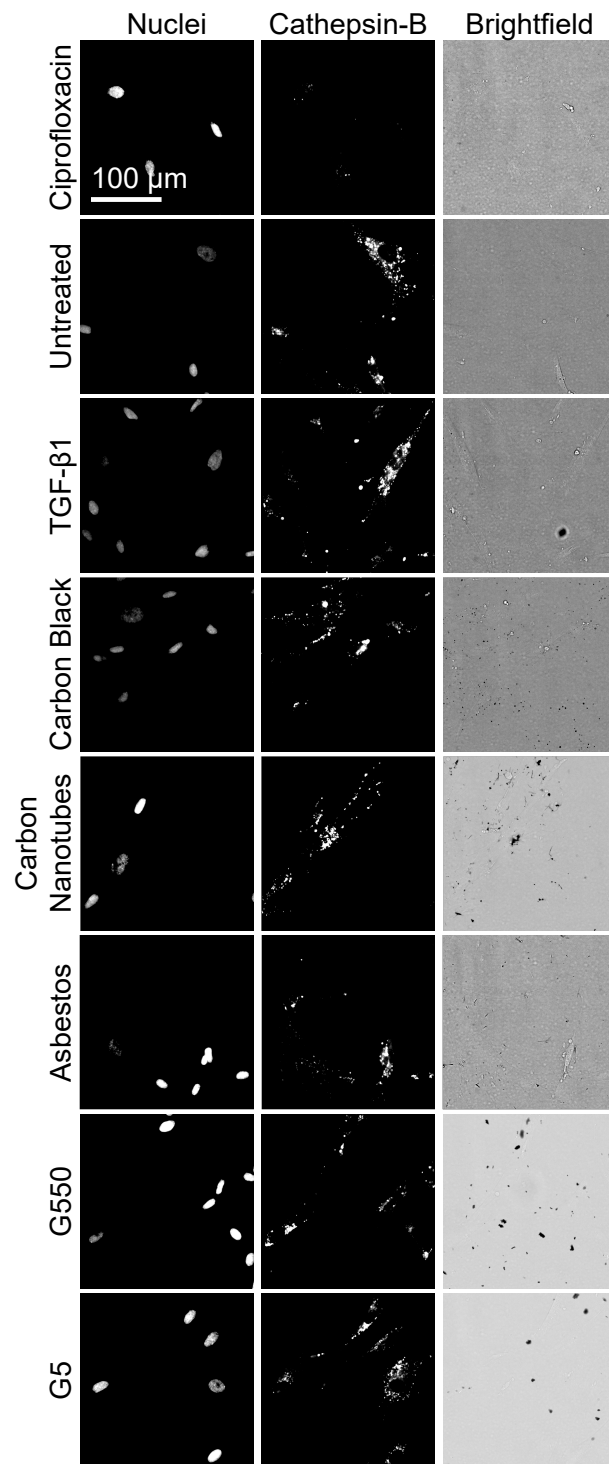


Figure 2.5: Punctate Cathepsin-B Suggests Undamaged Lysosomes

High concentration ciprofloxacin treatment induces lysosomal damage and release of cathepsin-B stain. The release of cathepsin-B into the cytoplasm is observed as low intensity, diffuse signal. Healthy, punctate lysosomes containing cathepsin-B are observed in every other condition after 24 hours. Scale bar = 100 μm .

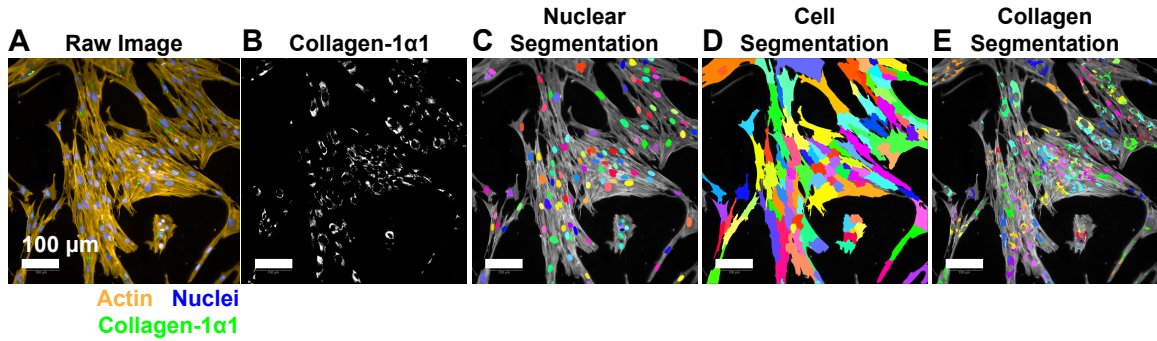


Figure 2.6: Collagen-1 α 1 Image Analysis Paradigm.

(A) IMR-90 fibroblast were cultured for 72 hours, fixed, and labeled with a collagen-1 α 1 antibody. In order to quantify collagen-1 α 1, raw images were split into individual channel to analyze (B) collagen-1 α 1 channels. Nuclear images were (B) segmented using intensity thresholding and used as a “seed”. The central nuclear “seed” was expanded in all directions until it comes in contact with the barrier of another nuclear “seed” or reached the edge of the segmented filamentous actin channel. (C) Expanded regions, including their nuclei, were re-identified as cells. (D) Collagen-1 α 1 was segmented within individual cells was quantified to yield collagen-1 α 1 area.

lasts were imaged for lysosome and nanomaterial colocalization after 24-hour particle exposures. Fibroblasts contain intact, punctate lysosomes that do not colocalize with individual or aggregated particles (Figure 2.4). In order to confirm that observed lysosomes do not exclude damaged or leaking lysosomes, a cathepsin-B stain was utilized to observe lysosomal damage as a result of particle uptake. Fibroblasts treated with a high concentration of ciprofloxacin exhibited diffuse cathepsin-B staining with poor visibility of low-intensity staining and few intact lysosomal compartments. In contrast, all other conditions showed high-intensity cathepsin-B puncta (Figure 2.5). The presence of intact, undamaged lysosomes supports that any results of fibroblast to myofibroblast differentiation experiments are not driven by inflammasome activity or apoptosis associated with lysosomal leakage. Additionally, while epithelial cells and macrophages are known to exhibit high rates of endo- and phagocytosis, respectively, after particle exposure, fibroblast endocytosis of particles is greatly reduced in vivo and is also confirmed in this in vitro model on rigid substrates.

As a preliminary measure of population fibroblast to myofibroblast differentiation, we measured single-cell collagen abundance and calculated mean collagen per cell area. To do this, we developed an analysis pipeline that segmented nuclei, expanded each object from the nuclear center outward until the segmentation met another object or found the edge of the segmented actin region. The expanded region around each nucleus defined the cell. Within each cellular region, collagen-1 α 1 area was calculated as a sum of any pixel above secondary only intensity (Figure 2.6). Quantification of average collagen-1 α 1 area per cell exhibited an increasing trend in collagen-1 α 1 area after particle exposure that was diminished in untreated and carbon black conditions (Figure 2.7). Based on the quantification of collagen abundance, we hypothesized that the acute time point was too early to observe significant increases in collagen and that biomarkers of differentiation could detect changes at the population level.

Image Segmentation for p-Smad2/3 and α -SMA Biomarker Quantification

p-Smad2/3 and α -SMA biomarkers were imaged at 48-hour and 72-hour timepoints, respectively, in order to capture events within the TGF- β signaling pathway over time (Figure S2.32). Cells cultured on collagen-coated polystyrene bottom plates are imaged as a grid layout and subsequently stitched together for biomarker quantification and image analysis (Figure S2.31). Cells are stained with nuclear and filamentous actin morphological stains and labeled with antibodies for each biomarker. Figure S2.31 illustrates a portion of the image analysis pathway used to identify regions in which the two biomarkers are quantified. Raw images of nuclear staining (Figure 2.8A) are segmented to identify foreground, high-intensity objects (Figure 2.8B). These objects are filtered by size and shape to select a population of “real” nuclei (Figure 2.8C). A 20 μ m perinuclear region is generated around the nucleus, within which all quantification of fibroblast to myofibroblast differentiation biomarkers occurs (Figure 2.8D). In addition to biomarker quantification within segmented image regions (Figure 2.9A),

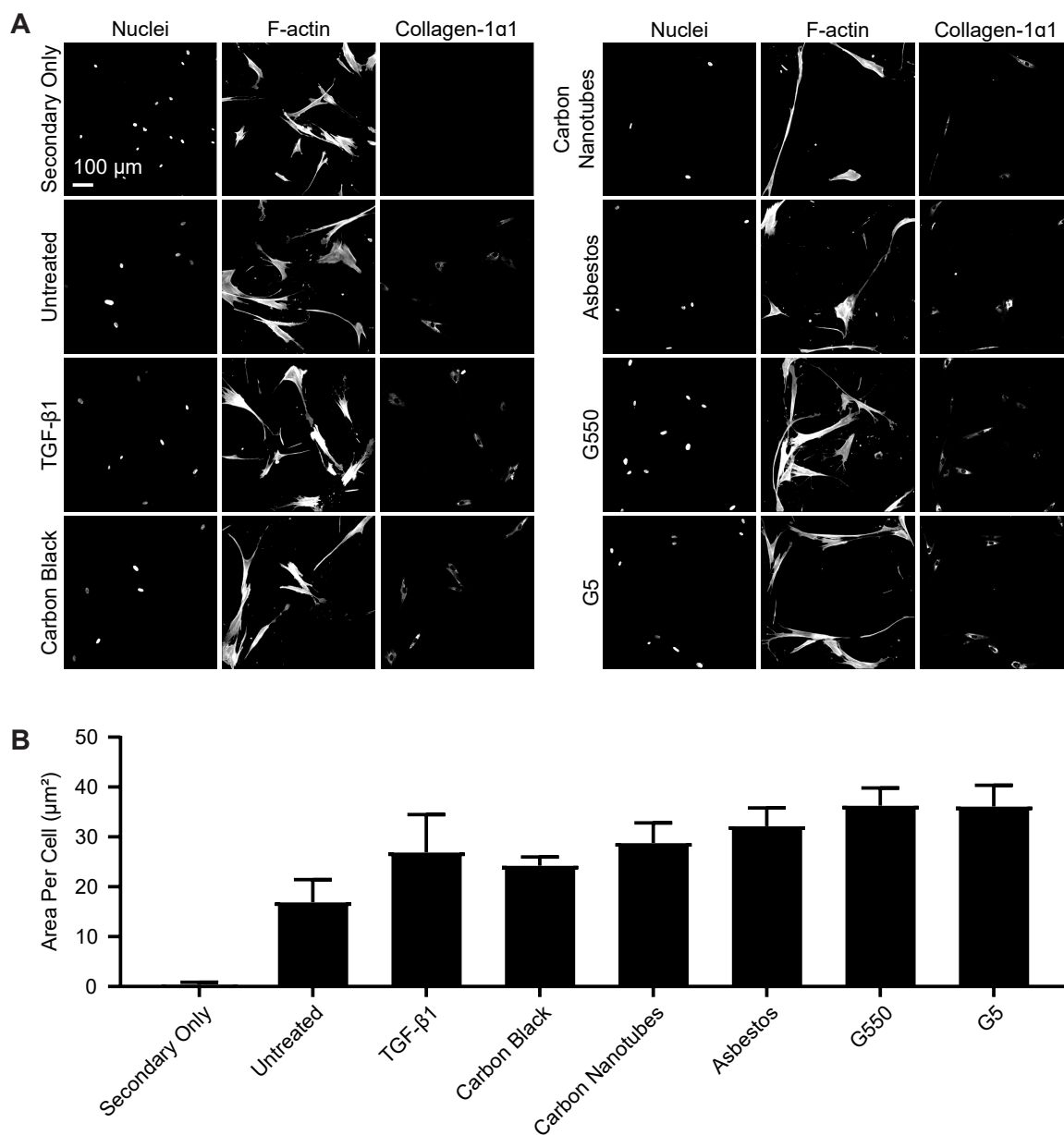


Figure 2.7: Particle exposure does not significantly alter collagen-1 α 1 abundance. IMR-90 fibroblasts were cultured for 72 hours and analyzed by a image analysis pipeline to quantify average collagen-1 α 1 per cell area. (A) Representative images of 72-hour, untreated, 5 ng/mL TGF- β 1, and 0.6 μ m/cm² particle exposure conditions, in addition to a secondary only negative antibody control. (B) Collagen-1 α 1 area per cell marginally increases after fibrous and graphene particle exposure. Statistics were calculated using a Kruskal-Wallis test with a Dunn's post-test.

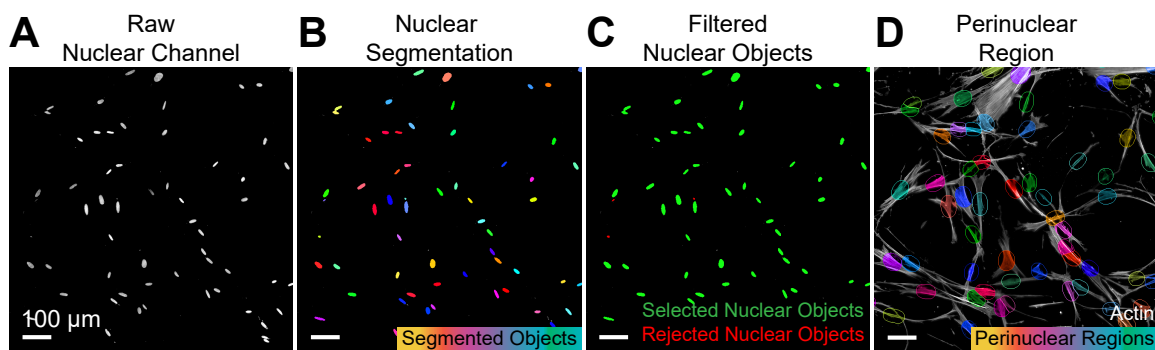


Figure 2.8: Nuclear and Perinuclear Region Segmentation

IMR-90 fibroblasts are grown in plates on rigid or soft substrates. (A) Confocal images of fibroblast nuclei are (B) segmented into nuclear objects by intensity thresholding. (C) Segmented objects are filtered by size and shape to identify a population of nuclei, around which a (D) perinuclear region is defined. Colors of segmented objects and perinuclear regions are not indicative of size or shape and is only intended to aid in the visualization of separate objects.

feature extraction techniques were employed to extract 28 numerical features (Table S2.3) from perinuclear segmented regions that describe biomarker and morphological endpoints, including nuclear shape and size, biomarker intensity, and biomarker, nuclear, and cytoskeletal features. The resulting dataset of values consists of a list of 28 single-cell features paired with categorical features, including treatment and fibroblast versus myofibroblast phenotypes based on biomarker area (Figure 2.9B). Finally, single-cell feature datasets are analyzed using multivariable regression, Factor Analysis of Mixed Data principle component analysis, and non-parametric ANOVA comparisons (Figure 2.9C).

The p-Smad2/3 transcription factor complex acts by translocating into the nucleus, producing a high-intensity nuclear signal in fibroblasts treated with TGF- β 1 (Figure S2.31C). In order to quantify this response, the p-Smad2/3 complex is only quantified within the segmented nuclear regions to identify actively differentiating cells (Figure S2.31D). α -SMA is a cytoskeletal protein incorporated into the cytoskeleton of myofibroblasts (Figure S2.31C). Quantification of α -SMA incorporation into the cytoskeleton is performed within the segmented perinuclear region (Figure

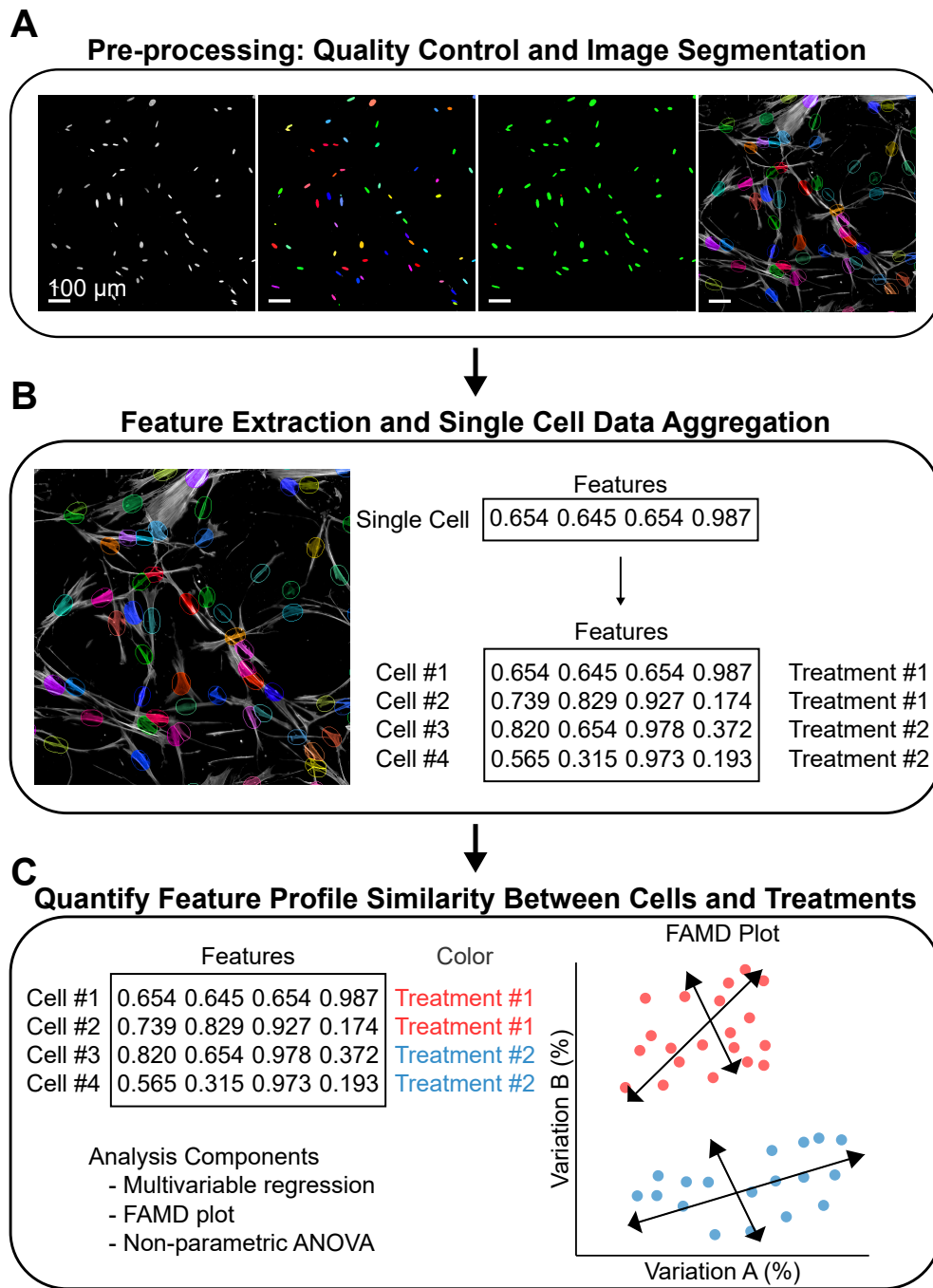


Figure 2.9: Biomarker Imaging and Multivariate Analysis
 (A) IMR-90 fibroblasts are imaged on a confocal microscope and segmented according to the description in Figure S2.31. (B) Within the nuclear and perinuclear regions, single-cell p-Smad2/3 and α -SMA feature profiles are collected that consist of numeric and categorical variables. (C) Multivariate analysis is performed for each rigid or soft substrate across IMR-90 fibroblast exposure conditions.

S2.31E). Attempts to identify membrane protein biomarkers for full cell segmentation were unsuccessful. During the generation of the perinuclear region, contact with another region stops region expansion. Segmentation follows similar principles to a Voronoi tessellation, which is defined as the segmentation of an image into regions through the expansion of regions from a set of chosen coordinates. In this case, the chosen coordinates are the central locations of each nucleus bounded by the nuclear region plus 20 μm (Figure S2.31B). Statistical evaluation of unimpeded 20 μm perinuclear region expansion versus 20 μm perinuclear expansion impeded by segmented actin regions showed no statistical difference in the percent α -SMA area per cell. Unimpeded nuclear expansion out to 20 μm was chosen for the final analysis. This turned out to be ideal for the incorporation of texture features in later analyses.

Reading Factor Analysis of Mixed Data (FAMD) Principle Component Analysis

In order to evaluate the observed heterogeneity further, categorical and numerical feature values were evaluated using the Factor Analysis of Mixed Data (FAMD) principal component method. As opposed to principle component analysis, which can only analyze numeric values, FAMD analysis is a mix between principal component analysis and multiple correspondence analysis (MCA), which is a principal component analysis that analyzes categorical value relationships. Single-cell values across each feature type were averaged across wells and plates for each condition to generate each set of dots colored by the condition. Each condition's resulting dot plots are described by a 95% confidence ellipse, which indicates that if the experiment were run 100 more times, 95 of the resulting values are predicted to fall within the ellipse. The average of data is denoted by the intersection of the two dotted lines, with translocation away from the center driven by variability in the first and second feature dimensions. Each feature in the dataset explains some percentage of variability in the data. The first

and second dimensions allow each condition value to inhabit a position with a relationship to all other points, with the distance between points describing the difference between the two values.

Rigid Substrate p-Smad2/3 and α -SMA Biomarkers

Initial image analysis and quantification of p-Smad2/3 and α -SMA biomarker abundance per cell was calculated using at the population level using image segmentation techniques (Figure 2.8)

Within segmented nuclear and perinuclear regions, 48-hour p-Smad2/3 complex translocation to the nucleus was utilized as a marker of active myofibroblast differentiation. Figure 2.10A shows an example of the actin and nuclear morphological stains, as well as a graphical illustration of the complex formation in the nucleus that was observed. TGF- β 1 exposure resulted in significant complex translocation from the cytoplasm to the nucleus. Additionally, an intermediate phenotype with fewer nuclear complexes was observed after carbon nanotube and asbestos exposure, with an additional graphene micro-sheet intermediate phenotype characterized by increased cytoplasmic complex formation. In order to separate the fibroblast and intermediate myofibroblast cell subpopulations, a threshold of >20% nuclear area positive for p-Smad2/3 complex was set. Using this threshold, fibroblasts have a consistent phenotype, while myofibroblasts exhibit population heterogeneity that is consistent between exposures, with the exception of TGF- β 1 exposures. This could be an indicator of variable particle exposures or variable fibroblast sensitivities to drivers of differentiation. Only TGF- β 1 significantly increased population differentiation compared to untreated conditions.

FAMD principle component analysis of p-Smad2/3 feature profiles on rigid substrates identify 1) separation between untreated and TGF- β 1 population phenotypes (Figure 2.11A) and overlapping population phenotypes, with increased population

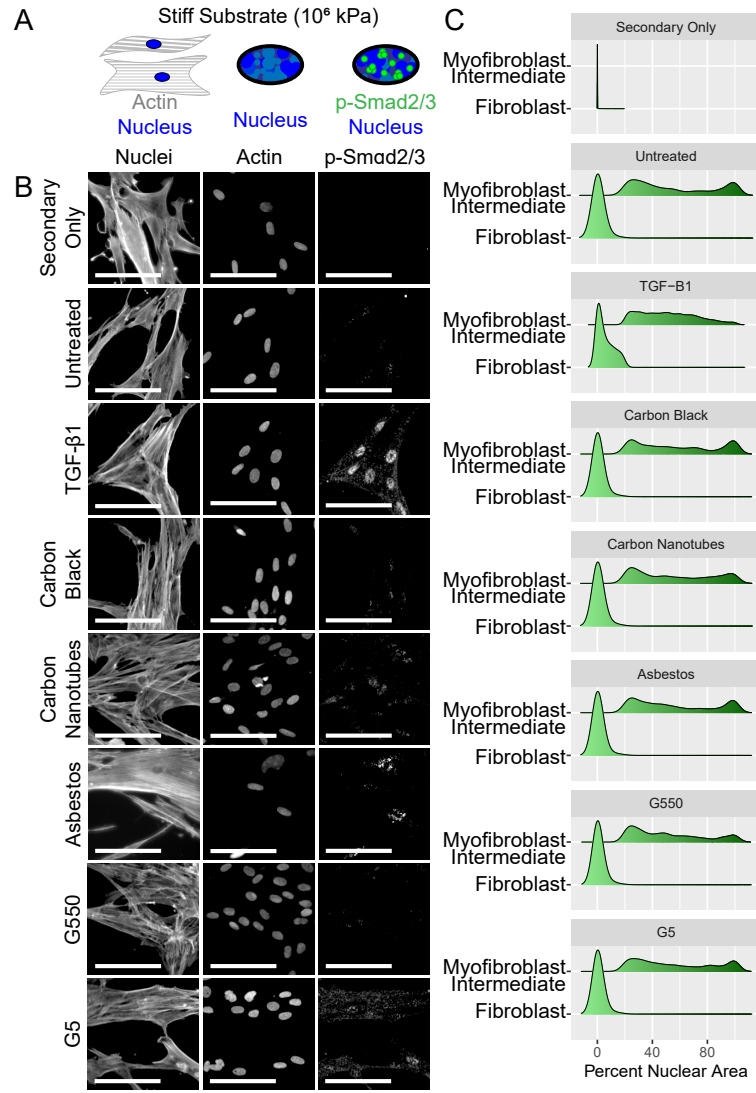


Figure 2.10: p-Smad2/3 Nuclear Translocation on Rigid Substrates

(A) IMR-90 fibroblasts are cultured on rigid plastic substrates coated in 5 $\mu\text{g}/\text{mL}$ collagen type 1. After 48 hours, fibroblasts are fixed and labeled for p-Smad2/3 complex. (A) Representative graphical illustration of actin, nuclear, and p-Smad2/3 complex labels. (B) TGF- β 1 exposure significantly increases p-Smad2/3 nuclear translocation compared to untreated conditions. Carbon nanotube and asbestos exposures exhibit intermediate nuclear translocation phenotypes, G5 graphene micro-sheets exhibit high cytoplasmic complex formation, and carbon black and G550 nano-sheet exposures resemble untreated conditions. (C) Subpopulations were created using a threshold of p-Smad2/3 nuclear area above $>20\%$. Fibroblast populations in all conditions are homogeneous, except for a secondary transitioning subpopulation after TGF- β 1 exposure. Actively differentiating intermediate myofibroblast cells exhibit heterogeneous populations under all conditions.

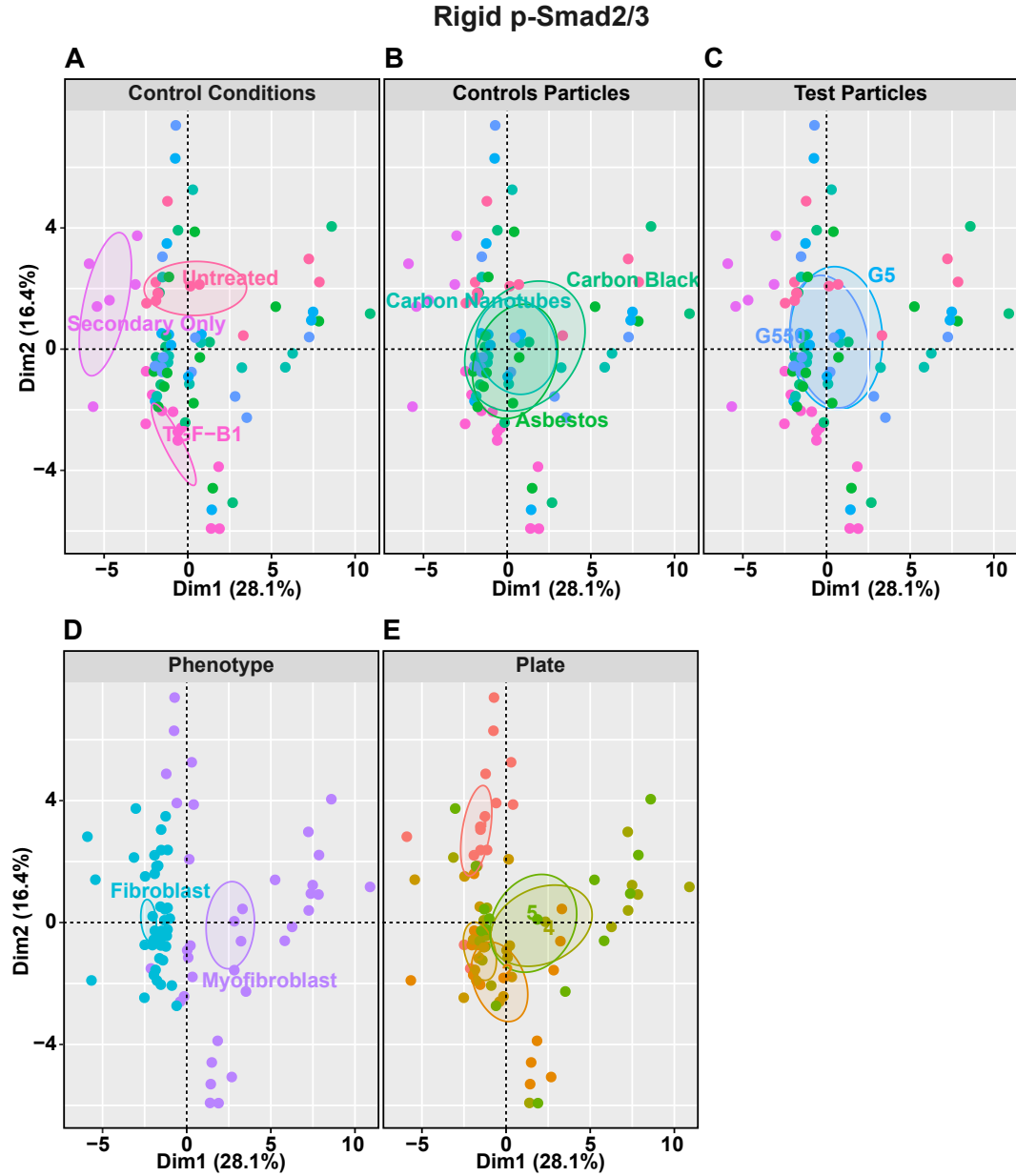


Figure 2.11: p-Smad2/3 Biomarker Features on Rigid Substrates
 IMR-90 fibroblasts fixed after 48 hours were labeled for p-Smad2/3, single-cell features were collected from nuclear and perinuclear regions, and single-cell data was processed using Factor Analysis of Mixed Data principle component analysis. Conditions across plates were averaged and analyzed to generate a 95% confidence ellipse for each condition, phenotype, and plate. (A) Control conditions show differences between TGF- β 1 and untreated conditions. (B) Carbon black, carbon nanotube, and asbestos exposures overlap but fall between untreated and TGF- β 1 population phenotypes. (C) G550 and G5 graphenes appear phenotypically similar to each other and other particle exposures. (D) Fibroblast and intermediate myofibroblast cell types have distinct phenotypes with (E) moderate plate variability.

heterogeneity, for all nanomaterial exposures that fall between untreated and TGF- β populations (Figure 2.11BC). Finally, cell phenotypes were highly differentiated in this analysis (Figure 2.11D), and plate variability was low (Figure 2.11E).

Figure 2.12A contains a graphical illustration of actin and nuclear morphological stains, as well as a graphical illustration of α -SMA myofibroblast biomarker incorporation into the cell cytoskeleton that was used to quantify changes in fibroblast populations on rigid substrates. Representative images across all conditions revealed a significant increase in α -SMA in after TGF- β 1 exposures and in small clusters after carbon nanotube, asbestos, and G5 micro-graphene exposures (Figure 2.12B). Quantification of the percent perinuclear region positive for α -SMA reveals similar fibroblast and myofibroblast profiles across conditions, with the exception of an extra fibroblast differentiating population in TGF- β 1 exposed populations (Figure 2.12C).

FAMD principle component analysis of α -SMA feature profiles on rigid substrates identify 1) some separation between untreated and TGF- β 1 population phenotypes (Figure 2.13A) and overlapping population phenotypes for all nanomaterial exposures that directly overlap between untreated populations (Figure 2.13BC), suggesting that cell populations do not recognize this biomarker cannot quantify nanomaterial exposures on stiff substrates or that phenotypic changes on stiff substrates without robust population shifts. Finally, cell phenotypes were highly differentiated in this analysis (Figure 2.13D), and plate variability was moderate (Figure 2.13E).

Fibrotic and TGF- β Signaling Pathway Gene Expression on Rigid Substrates

Despite detecting fibroblast to myofibroblast differentiation after TGF- β 1 or nanomaterial exposure, drivers of fibroblast to myofibroblast differentiation remained elusive due to targeted evaluation of TGF- β signaling pathway endpoints known to drive differentiation. Targeted fibrosis and TGF- β signaling pathway arrays aided in the

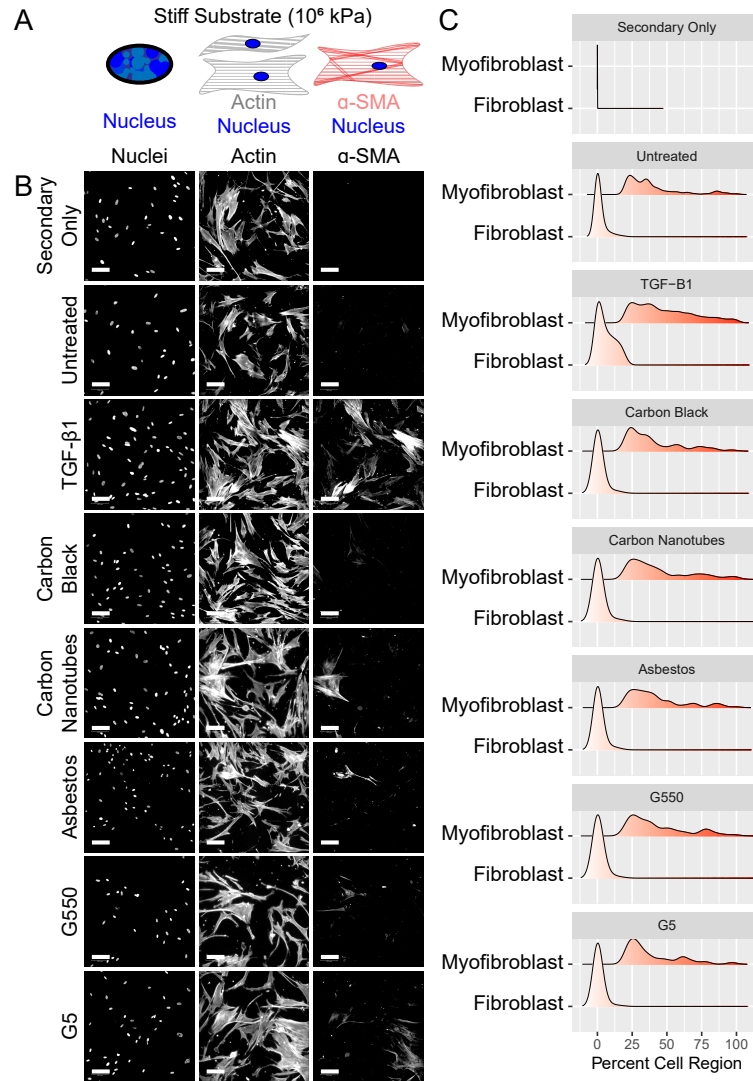


Figure 2.12: Cytoskeletal α -SMA on Rigid Substrates

(A) Representative graphical illustration of actin, nuclear, and α -SMA labels. (B) TGF- β 1 exposure significantly increased α -SMA biomarker expression compared to untreated conditions. Carbon nanotube, asbestos, and G5 graphene micro-sheet exposures cause differentiation in cell clusters, but carbon black and G550 nano-sheet exposures resemble untreated conditions. (C) Subpopulations were created using a threshold of α -SMA per cell area above >20%. Fibroblast populations in all conditions are homogeneous, except for a secondary transitioning subpopulation after TGF- β 1 exposure. Differentiated myofibroblasts exhibit heterogeneous populations under all conditions.

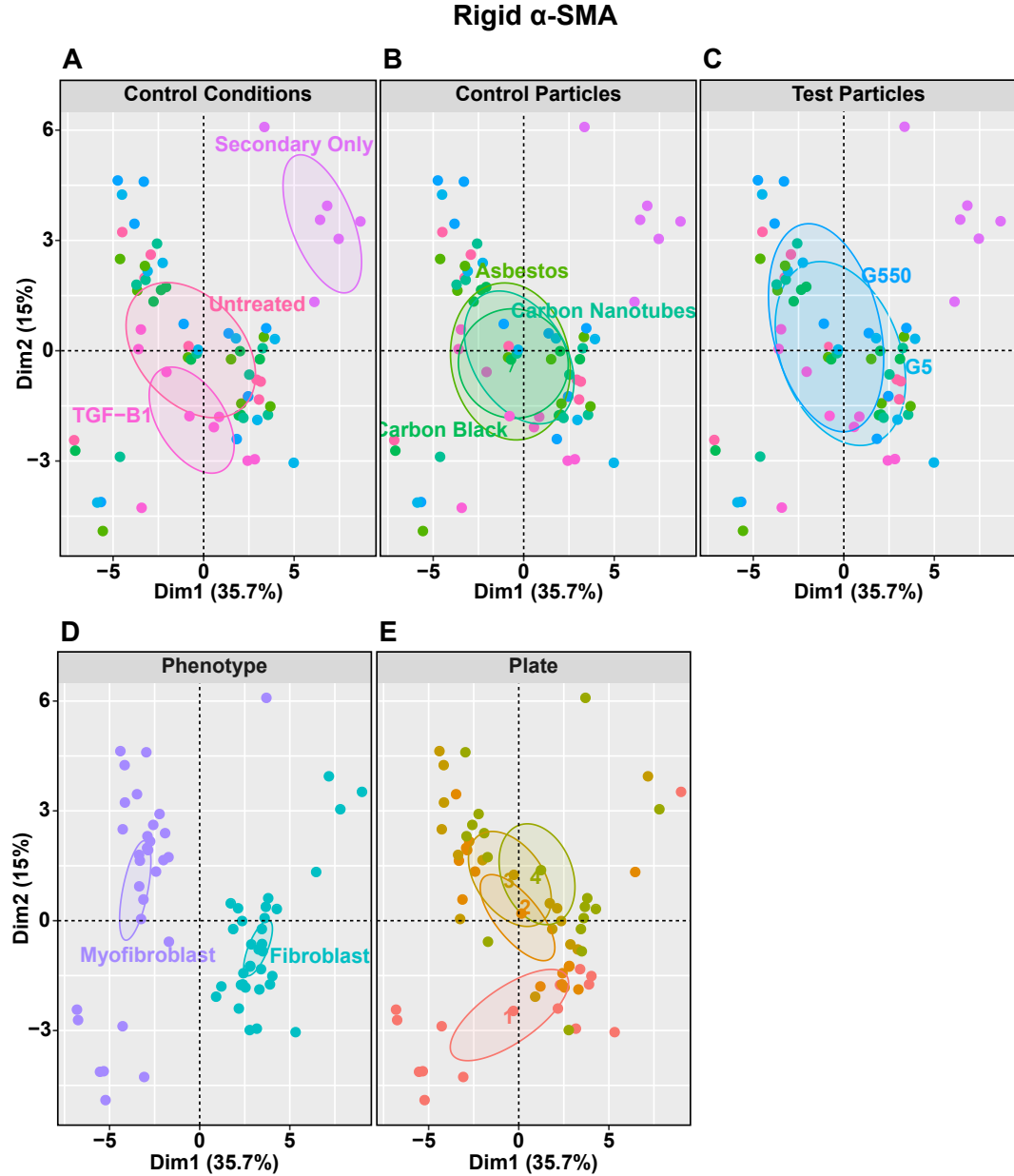


Figure 2.13: α -SMA Biomarker Features on Rigid Substrates
 IMR-90 fibroblasts fixed after 72 hours were labeled for α -SMA, single-cell features were collected from nuclear and perinuclear regions, and single-cell data was processed using Factor Analysis of Mixed Data principle component analysis. Conditions across plates were averaged and analyzed to generate a 95% confidence ellipse for each condition, phenotype, and plate. (A) Control conditions show differences between TGF- β 1 and untreated conditions. (B) Carbon black, carbon nanotube, and asbestos exposures overlap with untreated populations. (C) G550 and G5 graphenes appear phenotypically similar to each other and to other particle exposures. (D) Fibroblast and intermediate myofibroblast cell types have distinct phenotypes with (E) moderate plate variability.

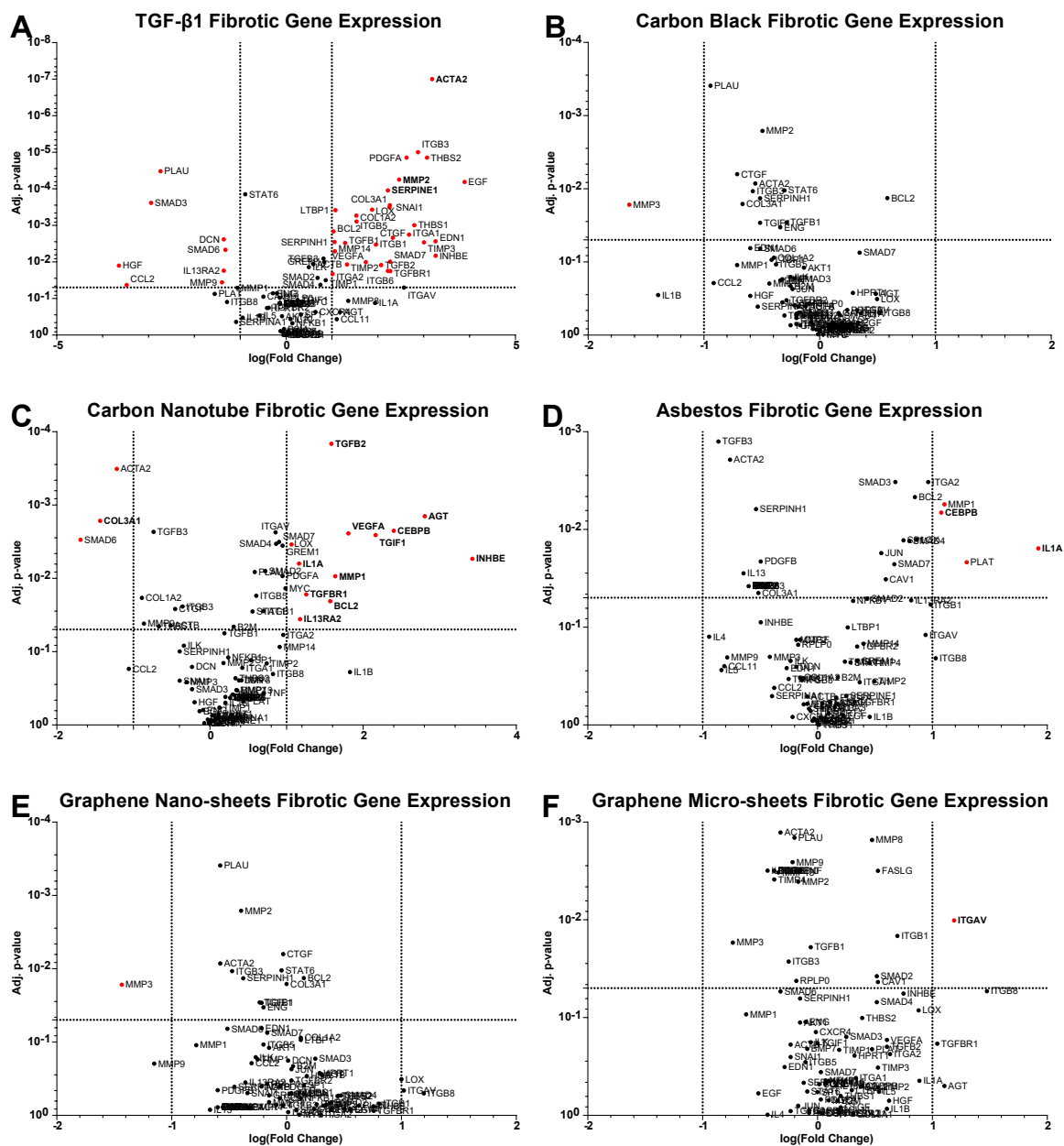


Figure 2.14: Fibrotic Gene Expression on Rigid Substrates

Of the significantly expressed genes profiled in Figure S2.35A, genes are highlighted (red) based on expression changes with two-fold up- or down-regulation (dotted lines at -1 and 1) and a p-value<0.05 (dotted line at y=0.05). The greatest changes in gene expression occur as a result of (A) TGF- β 1, (C) carbon nanotube, and (D) asbestos exposures as hypothesized. Few gene expression changes were observed in (B) carbon black and (E, F) graphene populations. Changes to key genes associated with the deposition of ECM, MMP and ECM remodeling, fibroblast to myofibroblast differentiation, and the TGF- β signaling pathways were identified. Genes shared between fibrotic and TGF- β signaling pathway arrays appear in bold.

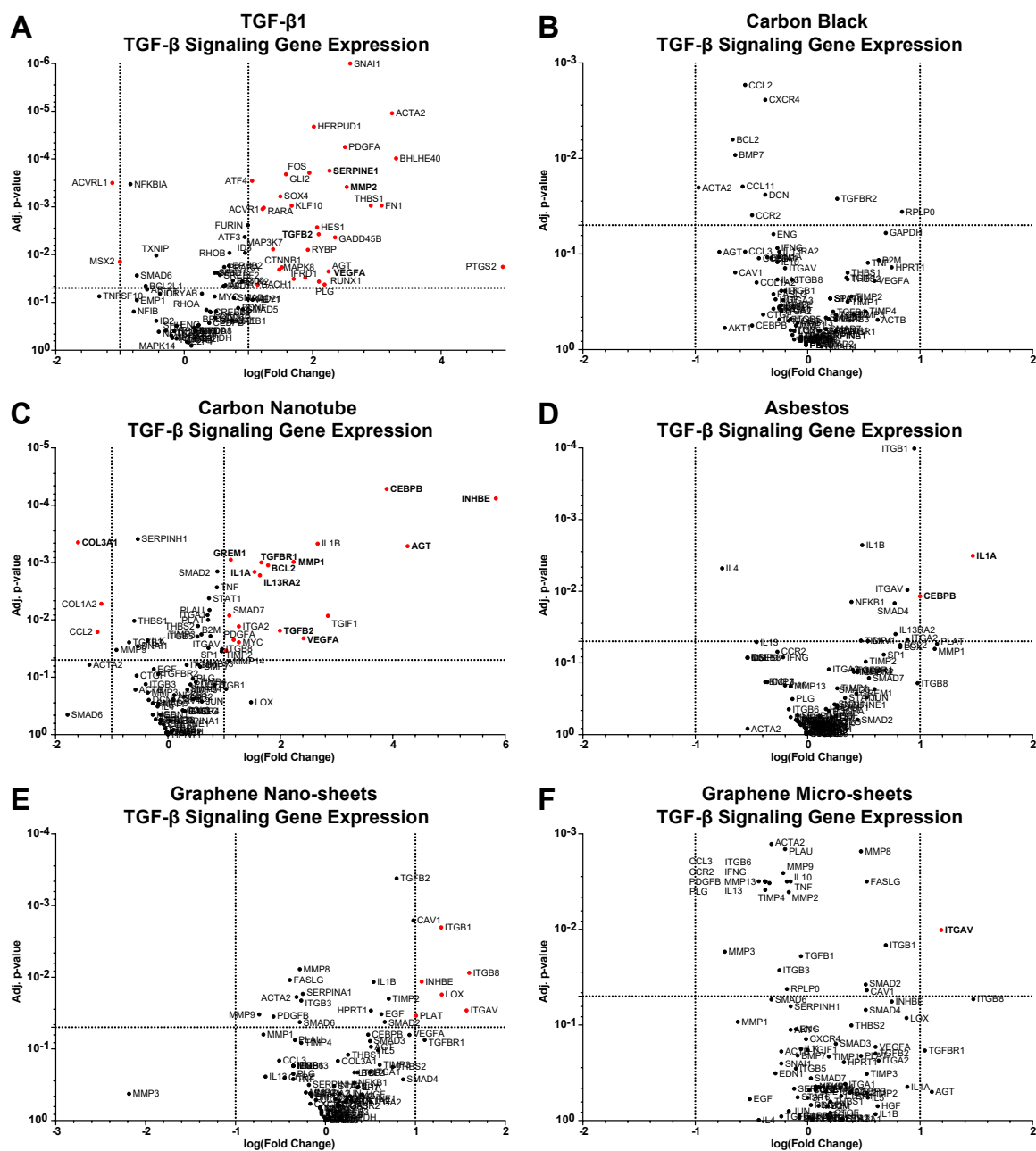


Figure 2.15: TGF- β Signaling Gene Expression on Rigid Substrates

Of the significantly expressed genes profiled in Figure S2.35B, genes are highlighted (red) based on expression changes with two-fold up- or down-regulation (dotted lines at -1 and 1) and a $p \leq 0.05$ (dotted line at 0.05). The greatest changes in gene expression occur as a result of (A) TGF- β 1 and (C) carbon nanotube exposures as hypothesized. Few gene expression changes were observed in (B) carbon black, (D) asbestos, and (E,F) graphene populations. Changes to key genes associated with the deposition of ECM, matrix metalloproteinase and ECM remodeling, and integrin precursors for the detection of ECM components. Genes shared between fibrotic and TGF- β signaling pathway arrays appear in bold.

evaluation of gene expression changes that likely drive large population transformation observed through FAMD analysis. The primary analysis of adjusted p-values revealed large numbers of significantly differentially expressed genes with fold changes >2 after 48-hour exposures compared to untreated fibroblast populations (Figure S2.35).

The use of targeted gene expression arrays supports the utility of this model as a screening tool. TGF- β 1 exposure results in significant changes in gene expression detected in both arrays and suggested activation of several pathways that mediate the fibrogenic potential of the resulting cell population. These traits are shared by carbon nanotube exposures in both arrays, with carbon nanotubes exposures inducing changes in gene expression that controls ECM deposition, matrix metalloproteinase and ECM remodeling, and integrin precursors for the detection of ECM components, cytoskeletal remodeling, growth and proliferation, and myofibroblast differentiation. Alternatively, asbestos exposure results in fewer gene expression changes in the TGF- β signaling pathway (Figure 2.15) compared to the fibrotic gene expression profile (Figure 2.14), suggesting a unique cell response driven by each nanomaterial's physicochemical characteristics.

Soft Substrate p-Smad2/3 and α -SMA Biomarkers

We hypothesized that fibroblast response to nanomaterials was being dampened by cell culture on rigid substrates. In order to test this hypothesis, we repeated the previous experiments on soft, 2 kPa tunable PA substrates that could mimic the stiffness of the human lung (Figure S2.36). Comparisons between collagen and fibronectin coatings for PA substrates to promote fibroblast adhesion showed no differences in cell response to TGF- β 1 or nanomaterial exposure (Figure S2.37). Collagen 1 was chosen as the final coating for all future experiments. Preliminary experiments confirmed fibroblast viability (Figure S2.38) and accurate image segmentation (Figure S2.39) on these newly integrated substrates.

Cell culture on soft substrates increase cytoplasmic p-Smad2/3 complex formation and have difficulty demonstrating nanomaterial effects.

Figure 2.16A contains a graphical illustration of actin and nuclear morphological stains and a graphical illustration of the intermediate myofibroblast biomarker p-Smad2/3 translocation into the nucleus that were used to quantify changes in fibroblast populations on soft substrates. Representative images across all conditions reveal a global increase in the cytoplasmic p-Smad2/3 complex formation that does not translocate into the nucleus (Figure 2.16B). Figure S2.40 contains representative higher resolution images of increased cytoplasmic p-Smad2/3 complex formation. Quantification of the percent nuclear area positive for p-Smad2/3 complex reveals similar fibroblast and myofibroblast profiles across conditions (Figure 2.16C).

FAMD principle component analysis of p-Smad2/3 feature profiles on soft substrates identify 1) decreased separation of untreated and TGF- β 1 population phenotypes (Figure 2.16A) and overlap between all nanomaterial exposures and untreated populations (Figure 2.16B), clustering of similar graphene morphologies (Figure 2.16C), and increased heterogeneity in carbon nanotube and graphene exposed populations. Finally, cell phenotypes were highly differentiated in this analysis (Figure 2.16D), and plate variability was low (Figure 2.16E). As a result of global increases in cytoskeletal p-Smad2/3, any subtle variance in p-Smad2/3 translocation to the nucleus is undetected and results in poor subpopulation phenotype definition.

2.4.3 Soft substrate α -SMA feature profiles identify differentiating populations of cells previously missed by population-level analysis.

Figure 2.18A contains a graphical illustration of actin and nuclear morphological stains, as well as a graphical illustration of the myofibroblast biomarker α -SMA in-

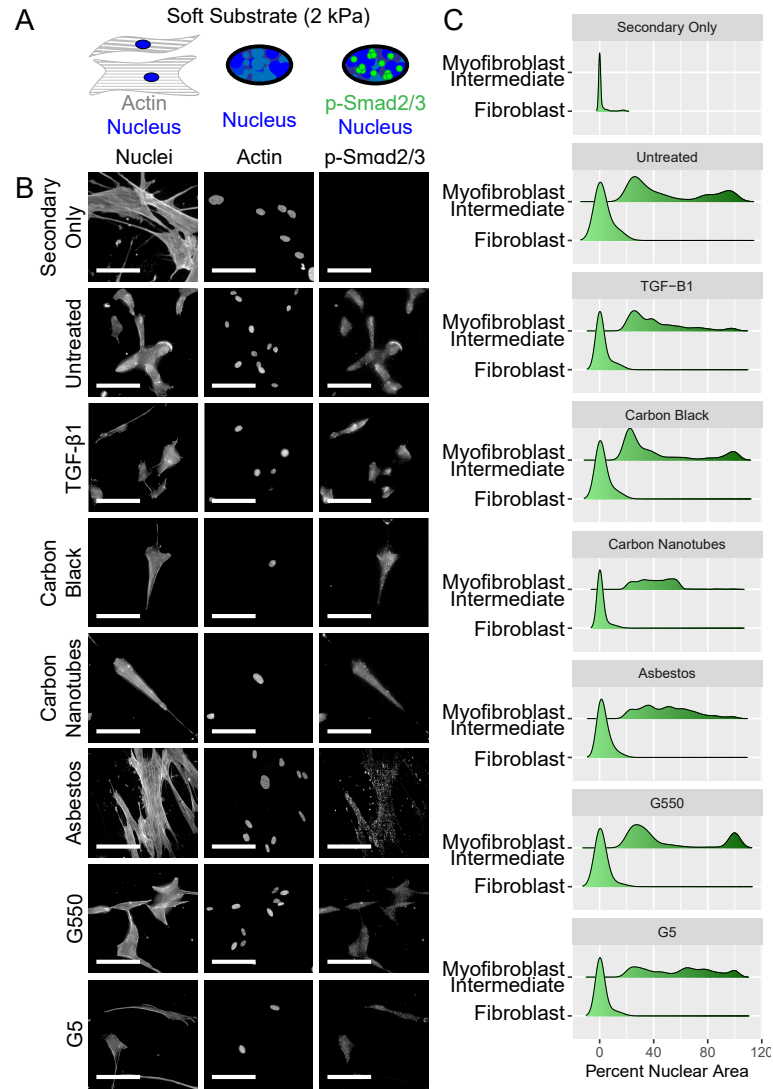


Figure 2.16: p-Smad2/3 Nuclear Translocation on Lung-mimicking Substrates (A) Representative graphical illustration of actin, nuclear, and p-Smad2/3 complex labels. (B) No exposures result in a significant population shift away from the untreated population. All fibroblasts on soft substrates increased cytoplasmic concentrations of p-Smad2/3 complex without increased p-Smad2/3 nuclear translocation. (C) Subpopulations were created using a threshold of p-Smad2/3 nuclear area above >20%. Fibroblast populations in all conditions are homogeneous, except for a secondary transitioning subpopulation after TGF- β 1 exposure. Actively differentiating intermediate myofibroblast cells exhibit heterogeneous populations under all conditions.

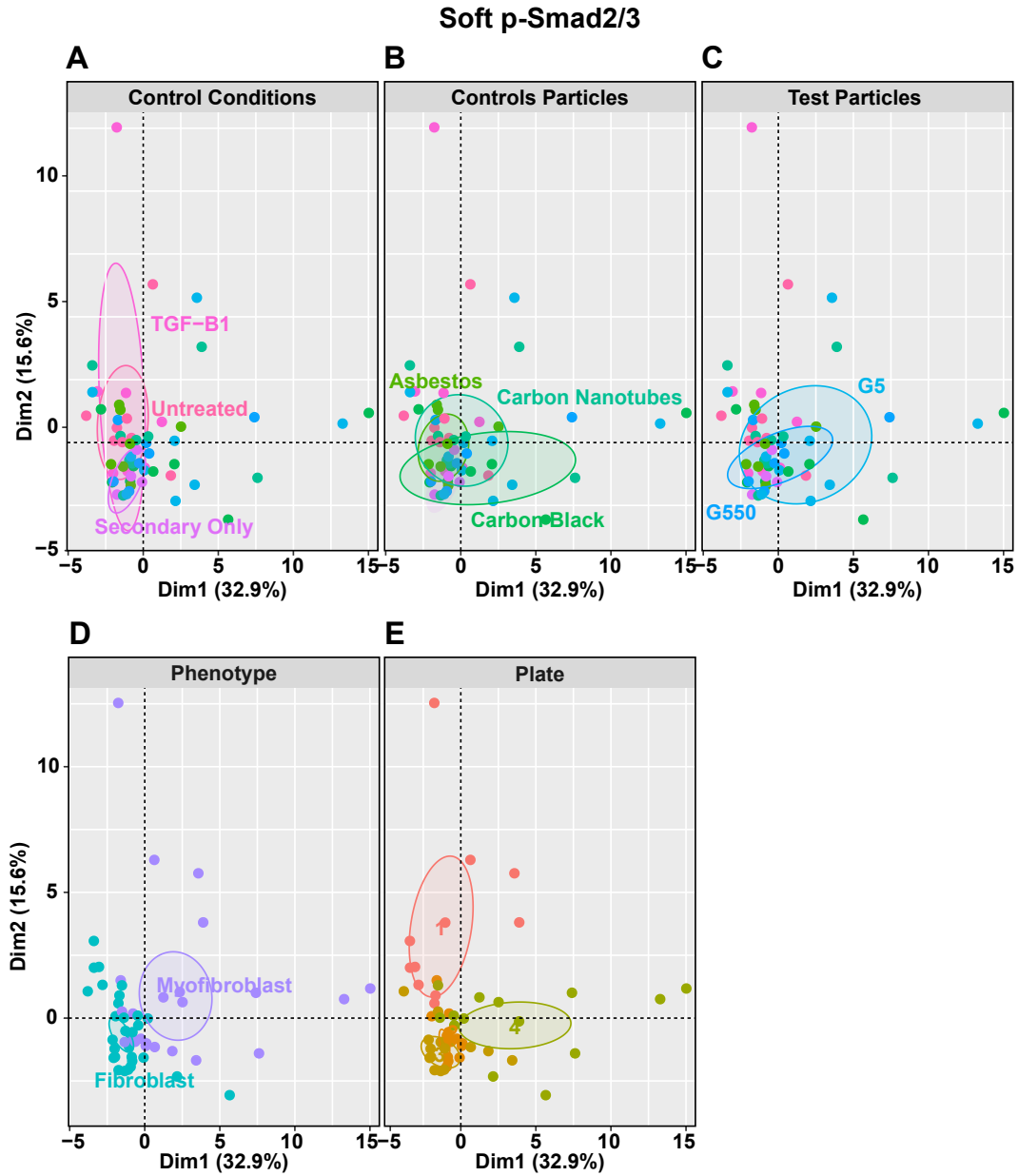


Figure 2.17: p-Smad2/3 Biomarker Features on Soft Substrates
 IMR-90 fibroblasts fixed after 48 hours were labeled for p-Smad2/3, and single-cell features were collected from nuclear and perinuclear regions. Single-cell data was processed using Factor Analysis of Mixed Data principle component analysis. Conditions across plates were averaged and analyzed to generate a 95% confidence ellipse for each condition, phenotype, and plate. (A) Control conditions show minimal differences between TGF- β 1 and untreated conditions. (B) Carbon black, carbon nanotube, and asbestos and (C) G550 and G5 graphene exposures overlap with each other and untreated population phenotypes. (D) Fibroblast and intermediate myofibroblast phenotypes are weakly separated with (E) moderate plate variability.

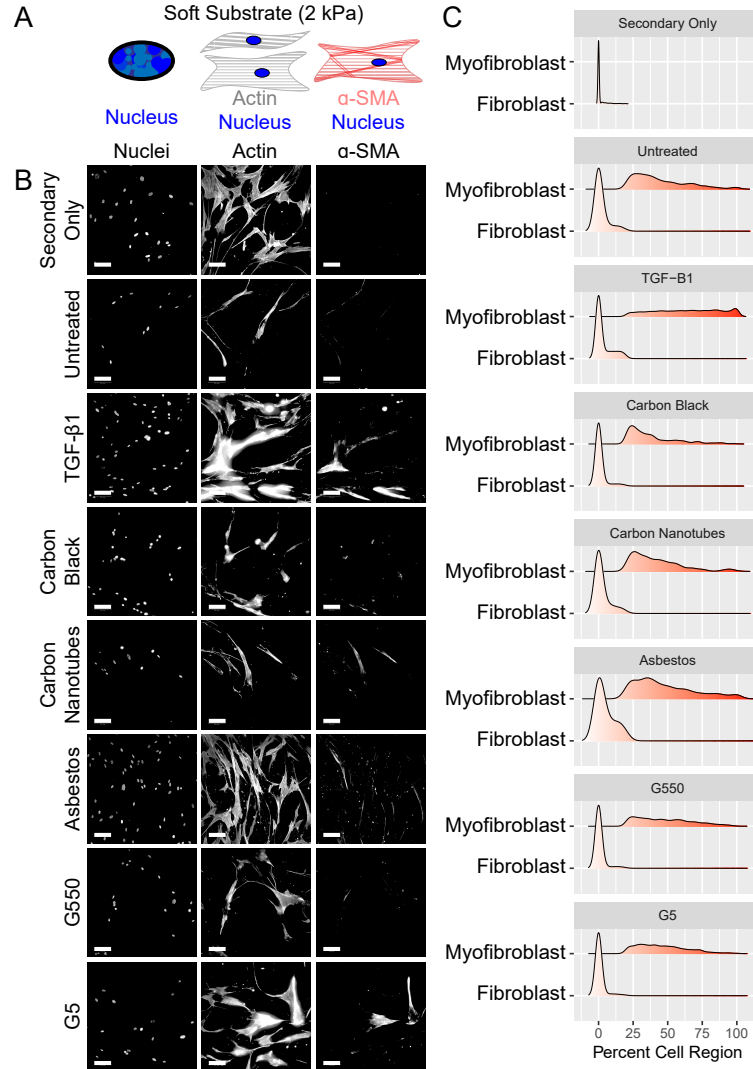


Figure 2.18: Cytoskeletal α -SMA on Lung-mimicking Substrates
 (A) Representative graphical illustration of actin, nuclear, and α -SMA labels. (B) TGF- β 1 exposure increased α -SMA biomarker expression compared to untreated conditions. Carbon nanotube, asbestos, and G5 graphene micro-sheet exposures drive differentiation in cell clusters, but carbon black and G550 nano-sheet exposures resemble untreated conditions. (C) Subpopulations were created using a threshold of α -SMA per cell area above $>20\%$. Fibroblast populations in all conditions are homogeneous, except for a secondary transitioning subpopulation after TGF- β 1 exposure. Differentiated myofibroblasts exhibit heterogeneous populations under all conditions.

corporation into the cell cytoskeleton that were used to quantify changes in fibroblast populations on soft substrates. Representative images of TGF- β 1 exposure reveals differentiation of some of the fibroblast population on soft substrates, and exposure to carbon nanotubes, asbestos, and G5 graphene micro-sheets induce clusters of myofibroblast differentiation (Figure 2.18B). Quantification of the percent area of the perinuclear region positive for α -SMA identifies new secondary differentiating fibroblast populations after carbon nanotube, asbestos, and TGF- β 1 exposures (Figure 2.18C).

FAMD principle component analysis of α -SMA feature profiles on soft substrates identify 1) decreased untreated and TGF- β 1 population heterogeneity (Figure 2.19A), transformation in carbon nanotube exposed populations (Figure 2.19B), clustering of similar graphene morphologies (Figure 2.19C), and increased heterogeneity in carbon nanotube and graphene exposed populations. Finally, cell phenotypes were highly differentiated in this analysis (Figure 2.19D), and plate variability was low (Figure 2.19E).

2.4.4 FAMD biplots of soft substrate α -SMA profiles identify changes in fibroblasts reminiscent of cytoskeletal and biomarker changes associated with fibrogenesis.

In order to interrogate drivers of the transformation of 95% confidence intervals generated in FAMD analysis, the top 10 features that explain the most population variability were extracted. p-Smad2/3 features profiles on rigid substrates were expected to detect variability driven by changes in biomarker texture and nuclear features. However, they detected cytoskeletal features, potentially detecting early actin remodeling in advance of myofibroblast differentiation (Figure 2.20A). p-Smad2/3 feature profiles on soft substrates are mostly explained by the first dimension and are predicted

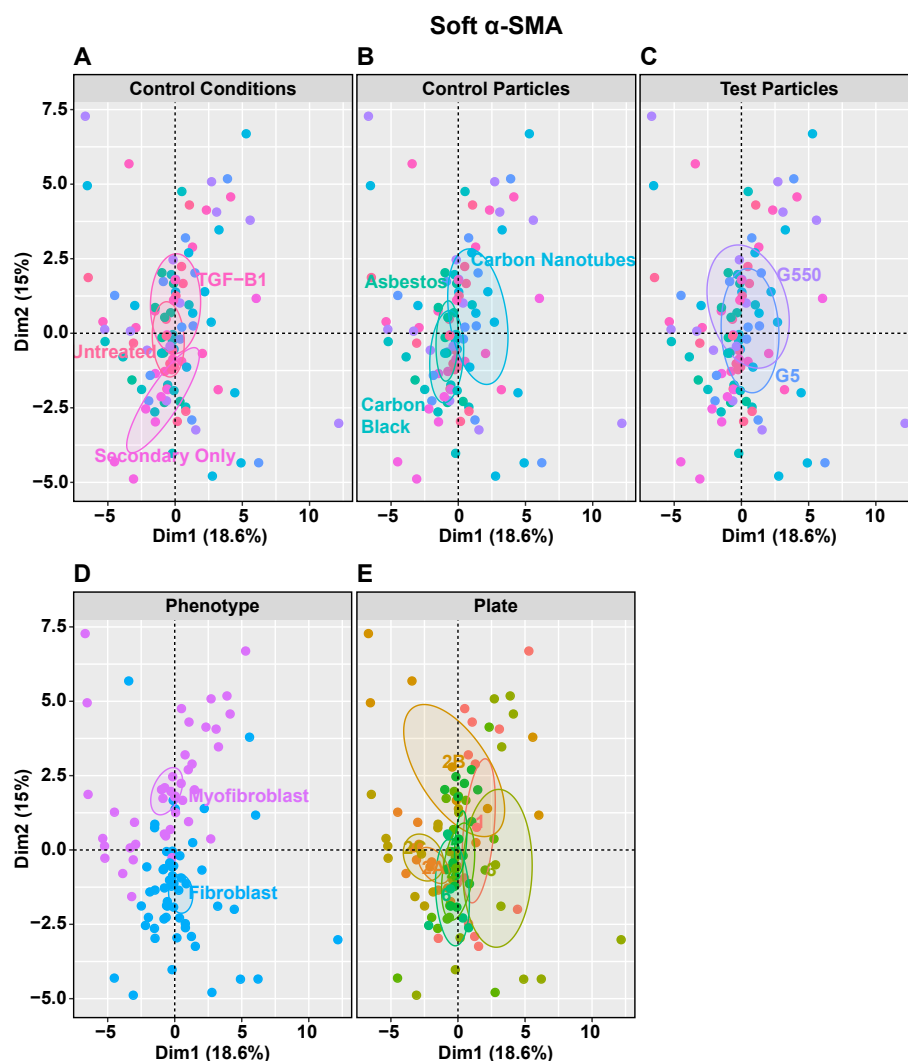


Figure 2.19: α -SMA Biomarker Features on Soft Substrates

IMR-90 fibroblasts fixed after 72 hours were labeled for α -SMA, single-cell features were collected from nuclear and perinuclear regions, and single-cell data was processed using Factor Analysis of Mixed Data principle component analysis. Conditions across plates were averaged and analyzed to generate a 95% confidence ellipse for each condition, phenotype, and plate. (A) Soft substrates are protective against TGF- β 1 exposure and result in less transformation of TGF- β 1 populations away from untreated conditions and decrease population heterogeneity. (B) Carbon black and asbestos exposures overlap with untreated populations. Carbon nanotube populations transform away from untreated populations but slightly overlap with TGF- β 1 populations. (C) G550 and G5 graphenes appear phenotypically similar to each other and fall between untreated and carbon nanotube populations. (D) Fibroblast and intermediate myofibroblast cell types have distinct phenotypes with (E) low plate variability.

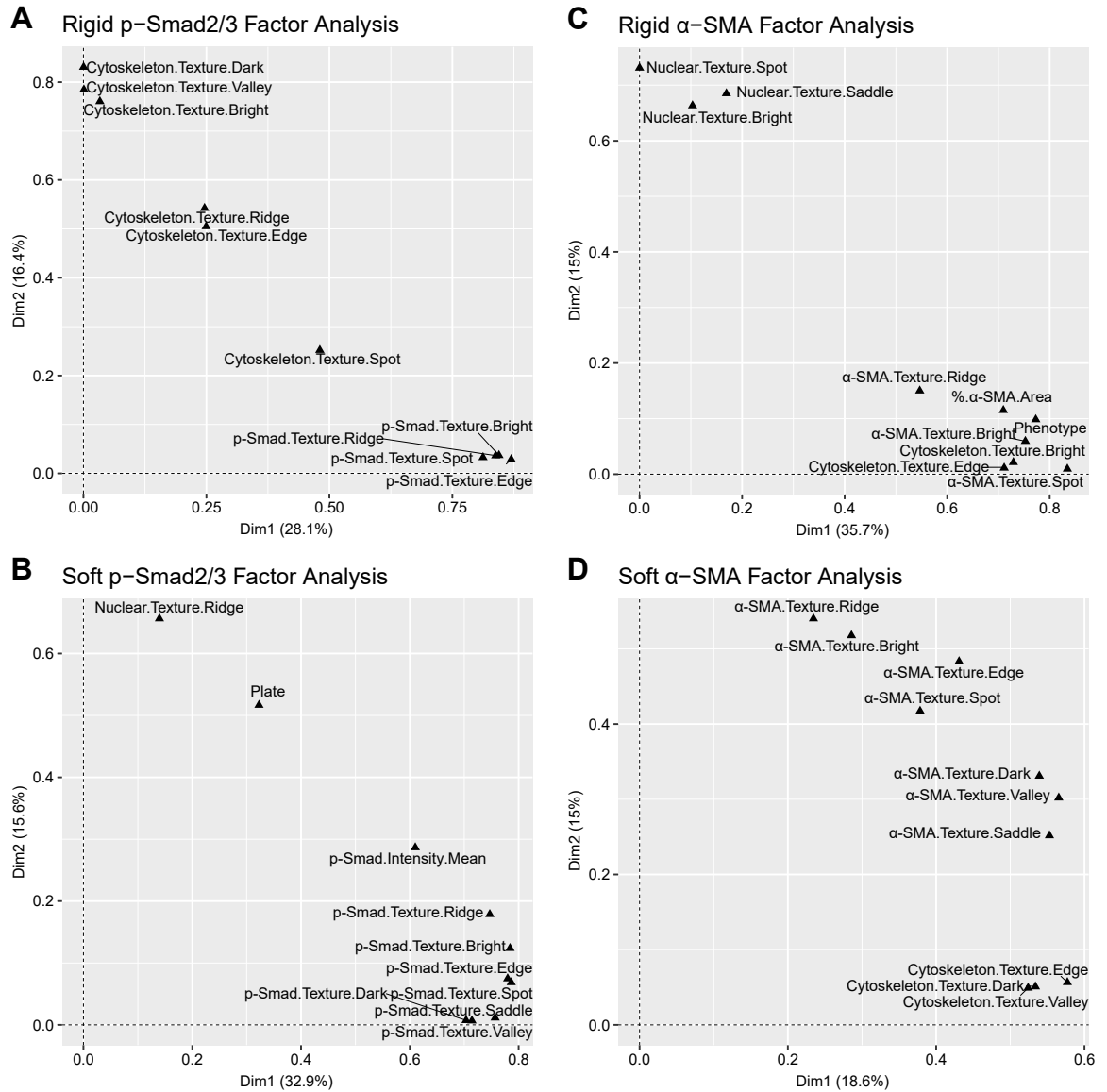


Figure 2.20: Top 10 FAMD Feature Relationships

(A) Rigid p-Smad2/3 feature profiles are most influenced p-Smad biomarker features. (B) Soft p-Smad2/3 feature profiles are most influenced by p-Smad2/3 features, likely as a result of the increased cytoplasmic p-Smad2/3 complex formation. (C) Rigid α-SMA feature profiles are most influenced by α-SMA features, with (D) soft α-SMA feature profiles heavily predicted by both cytoskeletal and α-SMA features.

by p-Smad2/3 texture features, likely due to the increased abundance of p-Smad2/3 complex throughout the entire fibroblast population (Figure 2.20B). α -SMA feature profiles on rigid substrates were expected to detect variability driven by changes in cytoskeletal and α -SMA texture features, but also detected nuclear features in addition to α -SMA and cytoskeletal features (Figure 2.20C). Alternatively, α -SMA feature profiles on soft substrates detect the hypothesized changes in cytoskeletal and α -SMA features (Figure 2.20D). If feature reduction were useful in decreasing sample processing due to tiered testing approaches of nanomaterials with known modes of action, the FAMD analysis technique could be used to identify the top 10 or fewer features that can predict changes and screen for nanomaterial toxicity. At present, the capabilities of this image analysis paradigm remain capable of identifying phenotypic changes driven by nuclear or cytoplasmic punctate or fibrous biomarker targets for incorporation into a variety of screening platforms.

2.4.5 Hierarchical clustering of α -SMA profiles separates nanomaterial exposures by geometry on soft substrates.

In order to evaluate how similar fibroblast populations are between conditions on different substrates, single-cell profiles across all conditions and substrates were evaluated using hierarchical clustering (Figure 2.21). p-Smad2/3 biomarker profiles successfully clustered untreated conditions and indicated that stiff substrates dampen fibroblast response to nanomaterials and make all nanomaterial exposures drive similar fibroblast phenotypes independent of nanomaterial stiffness or shape. Alternatively, soft substrate conditions increased recognition of nanomaterial exposures and promoted different population phenotypes. However, as a result of the global increase in cytoplasmic p-Smad2/3 complexes, soft substrate clustering of nanomate-

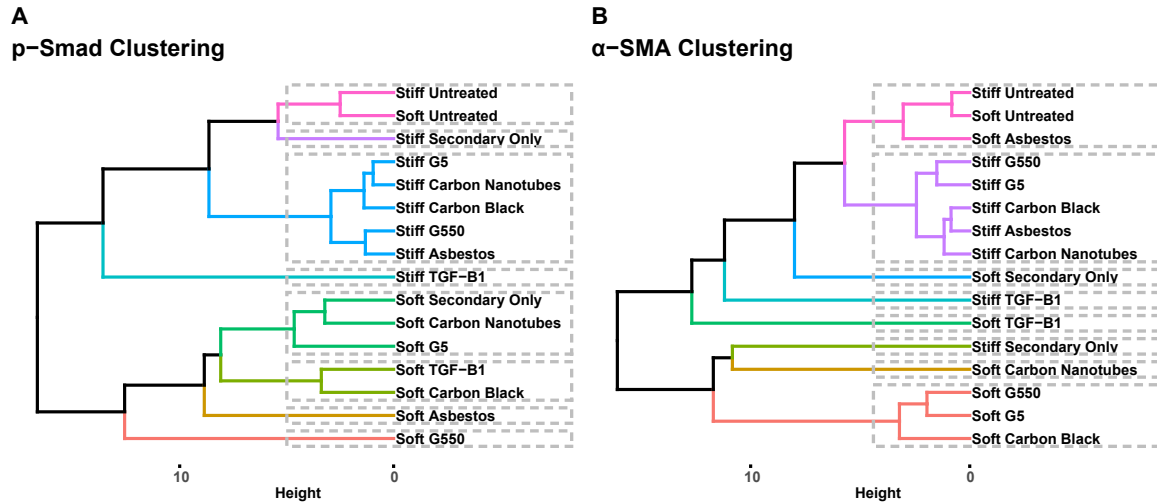


Figure 2.21: p-Smad2/3 and α -SMA Hierarchical Clustering on Rigid and Soft Substrates

Numeric features were averaged for every cell to give each cell a single value. All single-cell values within each condition on rigid and soft substrates were compared using hierarchical clustering to identify the most similar exposure and substrate conditions. Hierarchical clustering was performed on both (A) p-Smad and (B) α -SMA biomarker feature profiles. Conditions were artificially clustered into 8 groups and are indicated by gray, dotted line boxes. (16 total conditions would cluster into 8 groups if each condition clustered with itself independent of substrate.

rial geometries fails (Figure 2.21A). α -SMA feature profiles similarly cluster untreated conditions on both substrates together.

Additionally, untreated conditions are clustered with soft substrate asbestos populations. This could suggest a protective effect of asbestos exposure on soft substrates, or the acute time points evaluated by this biomarker are not sensitive enough to quantify the effects of asbestos exposure. α -SMA profiles after nanomaterial material exposure also cluster when evaluated on stiff substrates, again supporting that rigid cell culture outcompetes cell recognition of nanomaterial exposure. TGF- β 1 exposed populations are more similar under these biomarker profiles, and graphenes are clustered together. Finally, carbon nanotubes are separated from all other soft substrate exposures (Figure 21B). All in all, soft substrates allow cells to recognize

and display phenotypes associated with nanomaterial exposure that are not observed when nanomaterial toxicity is tested using traditional cell culture techniques.

2.5 Conclusions and Future Directions

Our initial experiments highlight two common toxicological problems, 1) the inability to quantify sub-cytotoxic changes, resulting in the need to test high concentrations of potential toxicants to detect toxicity, and 2) difficulty predicting chronic pathology in acute models, meaning that one must consider if a 5% change in a cell population is pathologically significant in the face of statistical insignificance. This thesis work proposes that image analysis and biostatistics techniques be applied more widely in toxicology as an economical alternative to flow cytometry, single-cell omics, and RNA-in situ hybridization (RNA-ISH). Our platform maintains the influence of mechano-stimuli until fixation, a stimulus that is lost during live-cell collection for flow cytometry. Image analysis can assess traits of population subsets after insoluble nanomaterial exposure without damaging or clogging machinery and circumvents the potential for damage to cells from nanomaterial collision at high flow rates. Secondly, single-cell omics is an impressive exploratory and targeted analysis tool but is expensive and time-consuming to analyze with a steep learning curve. Finally, RNA-ISH is invaluable for discrete targets of interest, but is expensive, has a higher learning curve, and typically needs to be confirmed at the protein level, requiring another set of experiments to confirm results. Our work supports the use of single-cell image analysis as a primary risk assessment tool to compare the potential adverse health impacts of emerging nanomaterials to well-characterized nanomaterial controls. Pairing image and multivariable analysis with other single-cell approaches offers added value to existing in vitro assay regulatory paradigms, especially where existing Adverse Outcome Pathways (AOPs) offer targeted biomarkers for study.

The scientific literature describes that fibroblasts contribute most significantly to the physical destruction of pulmonary architecture during pulmonary fibrosis. However, in our hands, the IMR-90 cell line was fickle, becoming senescent as early as passage 18 and actively secreting several globular proteins into the cell culture medium. IMR-90 fibroblasts were characterized and submitted to the ATCC in the 1970s and are best described as a normal fibroblast cell line. IMR-90 fibroblasts are not immortalized. The IMR-90 cell line has been stably cultured on tissue culture plastic for several generations, making them a readily-available, cost-effective, and un-mutated alternative to patient-derived or immortalized primary fibroblasts.

The main objective of this research is to increase the utility of previously established biomarkers of fibroblast to myofibroblast differentiation and incorporate them into a screening tool to predict nanomaterial toxicity in the human lung. Five key features of the experimental design were considered to maximize screening tool utility, an un-transformed human lung fibroblast cell line, a recombinant TGF- β 1-exposed control, toxic and non-toxic particle controls, a soft lung-mimicking substrate, and collagen coatings. By using recombinant TGF- β 1 as a control exposure to induce fibroblast to myofibroblast differentiation, we were able to use a 2D in vitro tool to identify sub-cytotoxic changes associated with a panel of nanomaterials of varying geometry, tensile strength, and previously characterized toxicity. This suggests that there is a similar driver of changes in fibroblasts shared by biopersistent nanomaterial exposures that is not observed after TGF- β 1 exposure, which we hypothesize is driven by mechanical interactions. In order to detect this subtle driver of differentiation that occurs in small sub-populations of fibroblasts in culture, the model system was developed in tandem with an image and statistical analysis pipeline that is capable of analyzing a wide array of cell phenotypes.

By performing image analysis using TGF- β signaling endpoints, we observed that α -SMA is the most sensitive endpoint. α -SMA was able to detect myofibroblast

differentiation across rigid and soft substrates that we failed to observe using the p-Smad2/3 biomarker. While α -SMA was capable of identifying sub-populations of differentiated cells on rigid and soft substrates, the variability in percent population differentiation between wells and plates limited our capability to identify statistically significant changes in populations associated with TGF- β or nanomaterial exposures. Through the development of the analysis pipeline for feature extraction in perinuclear regions of fibroblasts, we were able to bypass identification of the entire cell body and performed feature extraction from smaller segmented perinuclear regions. We relied on confidence intervals and FAMD principal component analysis to visualize subtle differences of feature profiles driven by subtle sub-population changes. Conventional approaches to principal component analysis use plotting of confidence intervals to evaluate the complete separation of clusters that denotes different phenotypic profiles. We utilized this technique to identify changes in sub-population differentiation and observe changes in population heterogeneity through changes in confidence interval size. Additionally, we observed the transformation of confidence intervals away from untreated and TGF- β 1 controls. Finally, we utilized hierarchical clustering to identify overall similarity in exposure profiles on stiff and soft substrates, observing that soft substrates allow cells to recognize fibrous carbon nanotubes and recognize sheet-like nanomaterials. The value in this technique lies in its potential for universal application across sample types and pathologies in the absence of complete single-cell segmentation, which is typically only possible for cuboidal cells with membrane labeling.

Matching variation in the fibroblast, intermediate myofibroblast, or myofibroblast biomarker profiles support the ability to compare between plating conditions. We used histograms to evaluate the distribution of biomarkers in discrete cell regions, as opposed to quantifying total percent population response, offering increased sensitivity to small population shifts. However, future work could incorporate the identifi-

cation and correlation of single-cell nanomaterial exposures to single-cell biomarker phenotypes. Additional research on the drivers of myofibroblast differentiation in cell clusters could identify secreted cytokine drivers and mechanical stimuli associated with local fibroblast activation during fibrogenesis and subtle changes to gene expression driven by nanomaterial exposure could be driven by other conditions apart from low, sub-cytotoxic nanomaterial exposure concentrations. Attempts to evaluate higher concentrations of nanomaterial exposure induced a decrease in overall significant differential pro-fibrotic gene expression (Figure S2.41 and Figure S2.42), which we hypothesize is prompted by increase apoptosis. Future experiments could evaluate the effects of higher concentrations to introduce apoptosis or necrosis into this model to increase complexity and evaluate a broader set of gene expression and morphological changes. The success of this analysis approach also lends itself to translating the analysis into 3D in vitro platforms. The application of this analysis paradigm in 3D in vitro platforms could assess more complex phenotypic changes driven by multicellular interactions and phenotype transience or adaptation at acute or chronic timepoints currently unavailable for analysis using traditional cell culture.

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2.7 Supplementary Information

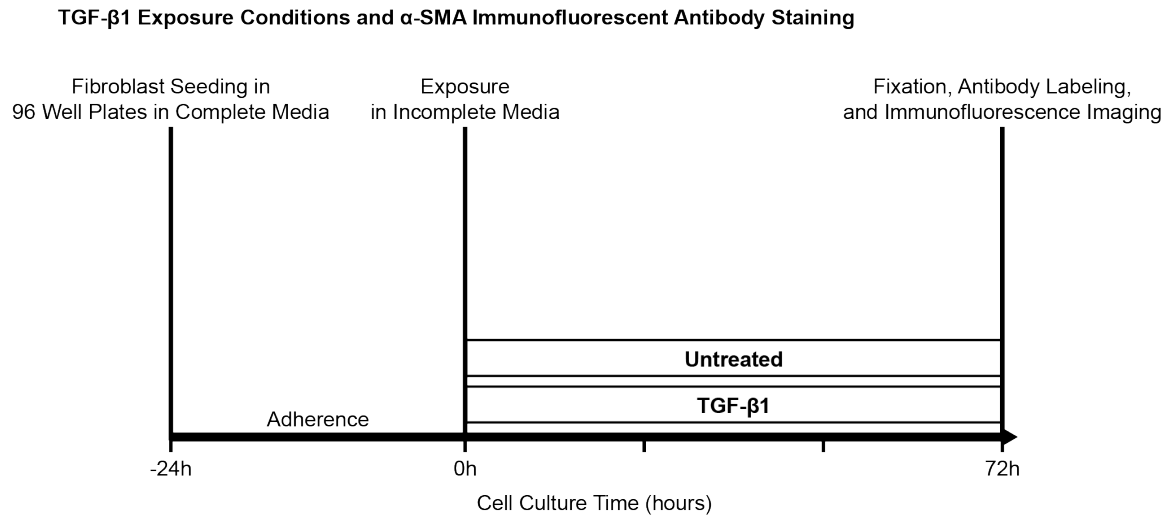


Figure 2.22: Experimental Timeline: TGF- β 1 Optimization Timeline

Sample and Origin	Length (μm)	Diameter (nm)	Fe content (wt. %)	Surface area (m ² /g)	Hydrodynamic size (μm)	Stiffness (kPa)
Carbon black: M120 Cabot Corp.	N/A	75	ND	35	0.273	N/A
Carbon Nanotubes: MWCNT-7 Mitsui & Co. ^[1]	11.7 ± 3.6	80 ± 18	< 0.2	35	1.379	3.6-63 x 10 ⁶ ^[2-6]
Crocidolite asbestos ^[1] UICC	2.8 ± 2.6	116 ± 112	22.2 ^[2]	9.1 ^[2]	ND	1.1-4.4 GPa ^[10]
Sample	Lateral dimension (μm)	Number of Layers	Thickness (nm)	Surface area (m ² /g)	Stiffness (monolayer) (kPa)	
G550: Graphene Cabot Corp.	0.550	25	8.5	25	ND	
G5: Graphene Graphene Supermarket	5	20	7	115	130 x 10 ⁶ ^[7] , modeled: 118-121 x 10 ⁶ ^[8, 9]	

Table 2.2: Nanomaterial Characterization with Additional Sources

Nanomaterial Characterization. N/A=not applicable. ND=undetermined. Works Cited 1. Sanchez, V.C., et al., A 3-dimensional in vitro model of epithelioid granulomas induced by high aspect ratio nanomaterials. Part Fibre Toxicol, 2011. 8: p. 17. 2. Yu, M.F., et al., Strength and breaking mechanism of multiwalled carbon nanotubes under tensile load. Science, 2000. 287(5453): p. 637-40. 3. Demczyk, B.G., et al., Direct mechanical measurement of the tensile strength and elastic modulus of multiwalled carbon nanotubes. Materials Science and Engineering, 2002. A 334.1-2: p. 173-178. 4. Mirmusavi, M.H., et al., Evaluation of physical, mechanical and biological properties of poly 3-hydroxybutyrate-chitosan-multiwalled carbon nanotube/silk nano-micro composite scaffold for cartilage tissue engineering applications. Int J Biol Macromol, 2019. 132: p. 822-835. 5. Yu, M.-F., et al., Tensile loading of ropes of single wall carbon nanotubes and their mechanical properties. Physical review letters, 2000. 6. Xie, S., Mechanical and physical properties on carbon nanotube. The Journal of physics and chemistry of solids, 2000. 61(7): p. 1153. 7. Liu, F., Pingbing Ming, and Ju Li., Ab initio calculation of ideal strength and phonon instability of graphene under tension. Physical review letters. 2007. 8. Lee, C., et al., Measurement of the elastic properties and intrinsic strength of monolayer graphene. Science, 2008. 321(5887): p. 385-8. 9. Roopam Khare, S.L.M., Jeffrey T. Paci, Sulin Zhang, Roberto Ballarini, George C. Schatz, Ted Belytschko, Coupled quantum mechanical/molecular mechanical modeling of the fracture of defective carbon nanotubes and graphene sheets. Phys. Rev., 2007. 10. Virta, Robert L., Asbestos: Geology, Mineralogy, Mining, and Uses. U.S. Department of the Interior, U.S. Geological Survey.

Feature Type	Features	Feature Type	Features
Nuclear Morphology	1. Nuclear Area (μm)	Nuclear Morphology	1. Nuclear Area (μm)
	2. Nuclear Roundness		2. Nuclear Roundness
α-SMA Morphology	3. α -SMA Region Area	p-Smad2/3 Morphology	3. p-Smad2/3 Region Area
α-SMA Intensity Features	4. α -SMA Intensity Contrast	p-Smad2/3 Intensity Features	4. p-Smad2/3 Intensity Contrast
	5. α -SMA Intensity Mean		5. p-Smad2/3 Intensity Mean
	6. α -SMA SP* Intensity Contrast		6. p-Smad2/3 SP* Intensity Contrast
	7. α -SMA SP* Intensity Mean		7. p-Smad2/3 SP* Intensity Mean
α-SMA Textures	8. α -SMA Texture Bright	p-Smad2/3 Textures	8. p-Smad2/3 Texture Bright
	9. α -SMA Texture Dark		9. p-Smad2/3 Texture Dark
	10. α -SMA Texture Edge		10. p-Smad2/3 Texture Edge
	11. α -SMA Texture Hole		11. p-Smad2/3 Texture Hole
	12. α -SMA Texture Ridge		12. p-Smad2/3 Texture Ridge
	13. α -SMA Texture Saddle		13. p-Smad2/3 Texture Saddle
	14. α -SMA Texture Valley		14. p-Smad2/3 Texture Valley
Nuclear Textures	15. Nuclear Texture Bright	Nuclear Textures	15. Nuclear Texture Bright
	16. Nuclear Texture Dark		16. Nuclear Texture Dark
	17. Nuclear Texture Edge		17. Nuclear Texture Edge
	18. Nuclear Texture Hole		18. Nuclear Texture Hole
	19. Nuclear Texture Ridge		19. Nuclear Texture Ridge
	20. Nuclear Texture Saddle		20. Nuclear Texture Saddle
	21. Nuclear Texture Valley		21. Nuclear Texture Valley
Cytoskeletal Textures	22. Cytoskeleton Texture Bright	Cytoskeletal Textures	22. Cytoskeleton Texture Bright
	23. Cytoskeleton Texture Dark		23. Cytoskeleton Texture Dark
	24. Cytoskeleton Texture Edge		24. Cytoskeleton Texture Edge
	25. Cytoskeleton Texture Hole		25. Cytoskeleton Texture Hole
	26. Cytoskeleton Texture Ridge		26. Cytoskeleton Texture Ridge
	27. Cytoskeleton Texture Saddle		27. Cytoskeleton Texture Saddle
	28. Cytoskeleton Texture Valley		28. Cytoskeleton Texture Valley

Table 2.3: Fibroblast Feature Extraction From Segmented Regions

PA Stiffness	Final Acry Solution* (%)	Final Bis Solution* (%)	Water (μL)	Acrylamide (μL)	Bis (μL)	APS (μL)	TEMED (μL)	Total Volume (μL)
~2 kPa	3	0.2	161.5	15	20	2.5	1	200
~8.7 kPa	8	0.08	124.5	40	8	2.5	1	2.5

*Acry solution = 40% acrylamide (BioRad #1610140); Bis solution = 2% bis-acrylamide solution (BioRad #1610142)

Table 2.4: Polyacrylamide Gel Formulations

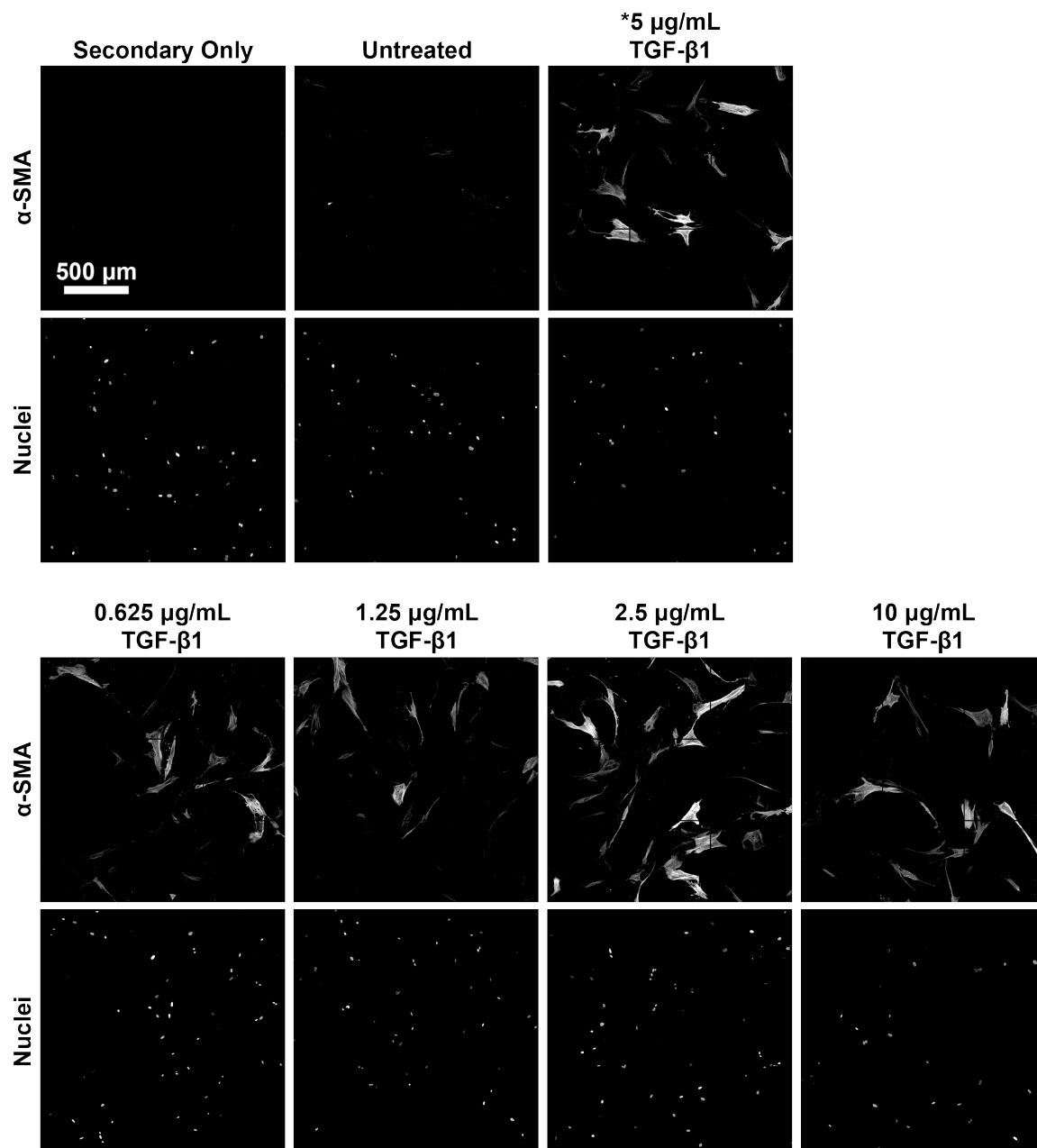


Figure 2.23: TGF-β1 Dose Curve Optimization

Primary IMR-90 fibroblasts were exposed to concentrations ranging from 0.625 - 10 μg/mL of recombinant TGFβ1, fixed at 72 hours, and labeled with α-SMA antibody for myofibroblast identification. 2.5 - 5 μg/mL of recombinant TGFβ1 produced consistent, high percentage differentiation without the cell death observed after exposure to 10 μg/mL of recombinant TGFβ1. Scale bar = 500 μm.

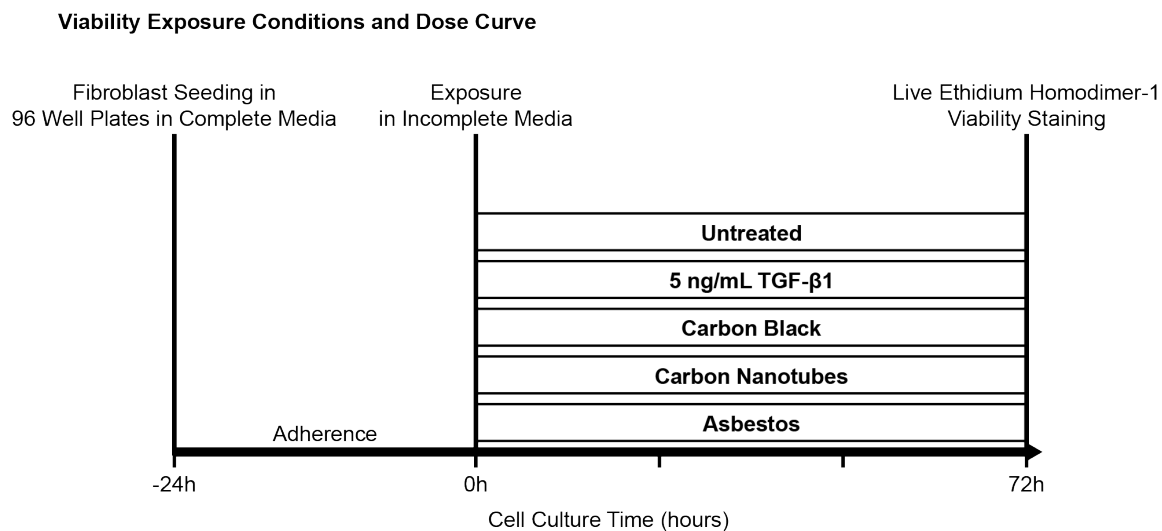


Figure 2.24: Experimental Timeline: Viability Dose Curve

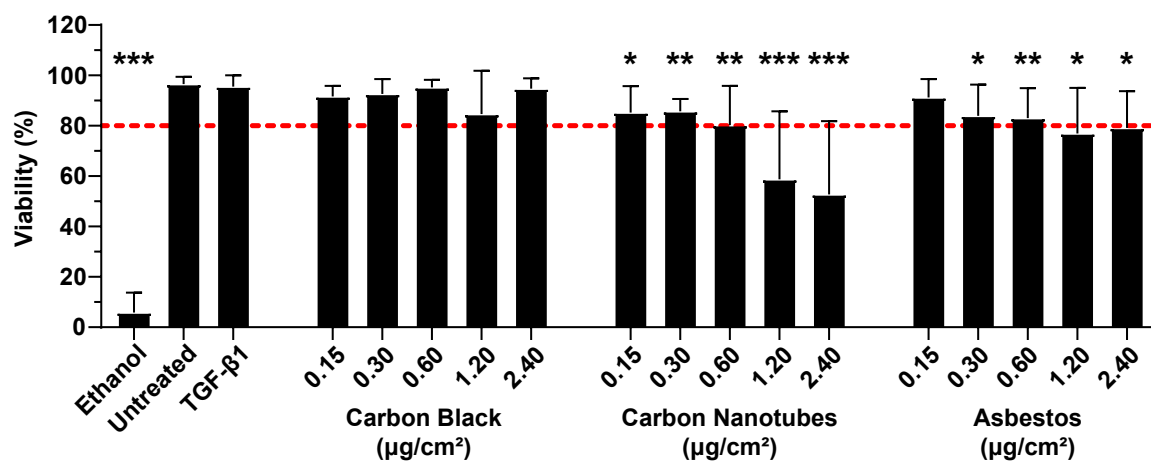


Figure 2.25: Determining Nanomaterial Exposure Concentrations Using Fibroblast Viability

Carbon nanotube and asbestos exposures significantly decrease IMR-90 fibroblast viability after a 72 hour exposure. $0.6 \mu\text{g}/\text{cm}^2$ carbon nanotube and asbestos exposures allow $>80\%$ viability and was the concentration chosen for future 2D experiments.

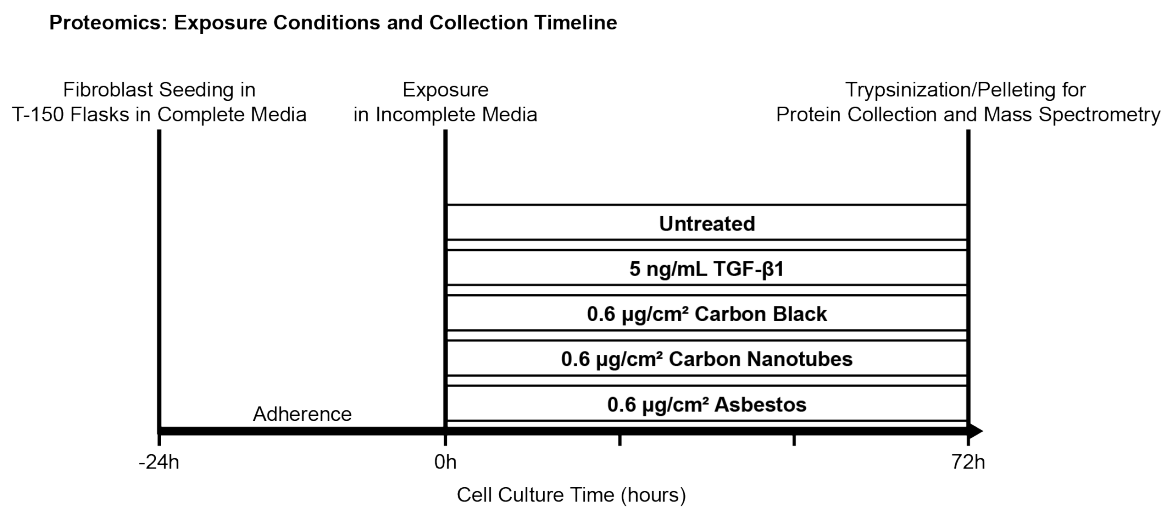


Figure 2.26: Experimental Timeline: Proteomics

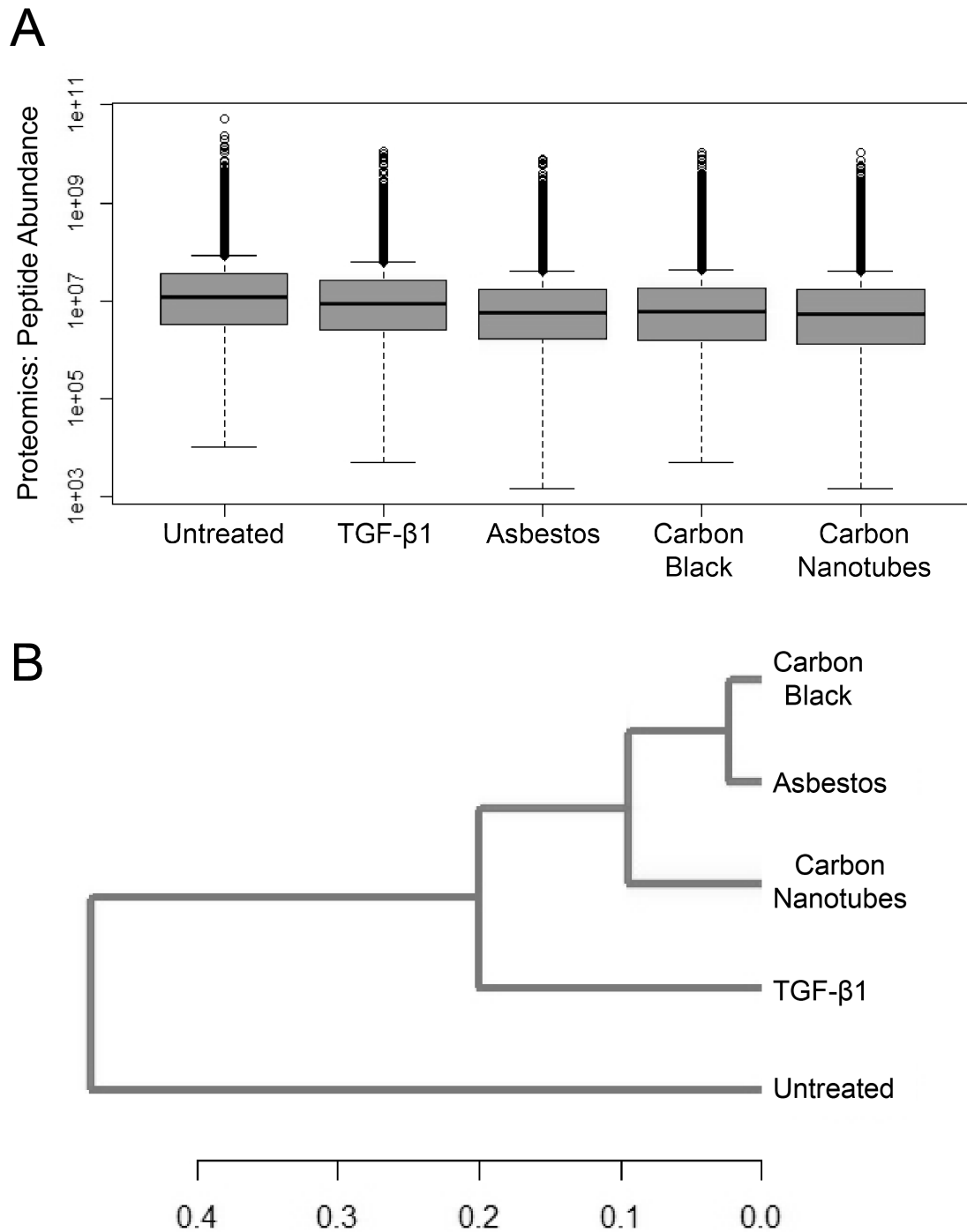


Figure 2.27: Proteomics: Normalized Peptide Abundance and Hierarchical Clustering
 Processed mass spectrometry data of 72 hour peptide abundance was median normalized. (A) Graphing median peptide abundance in decreasing order identifies a subtle decrease in overall peptide abundance after nanomaterial exposure, and (B) is confirmed by hierarchical clustering of the peptide profiles.

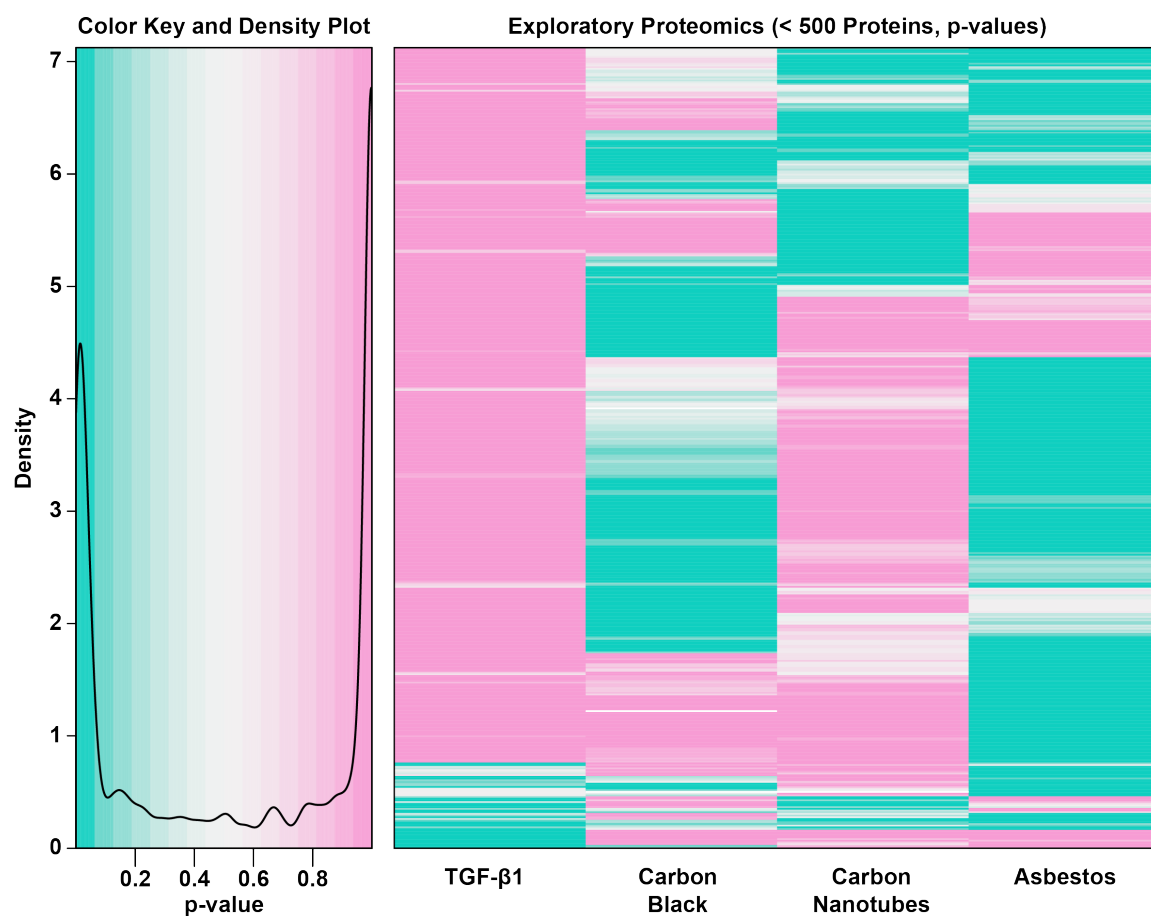


Figure 2.28: Significant Changes in Protein Abundance after 72 hours. Fold changes in protein abundance were evaluated by multiple t-test and corrected for multiple comparisons to yield adjusted p-values associated with each protein compared to untreated samples. p-value<0.05 is indicated in teal.

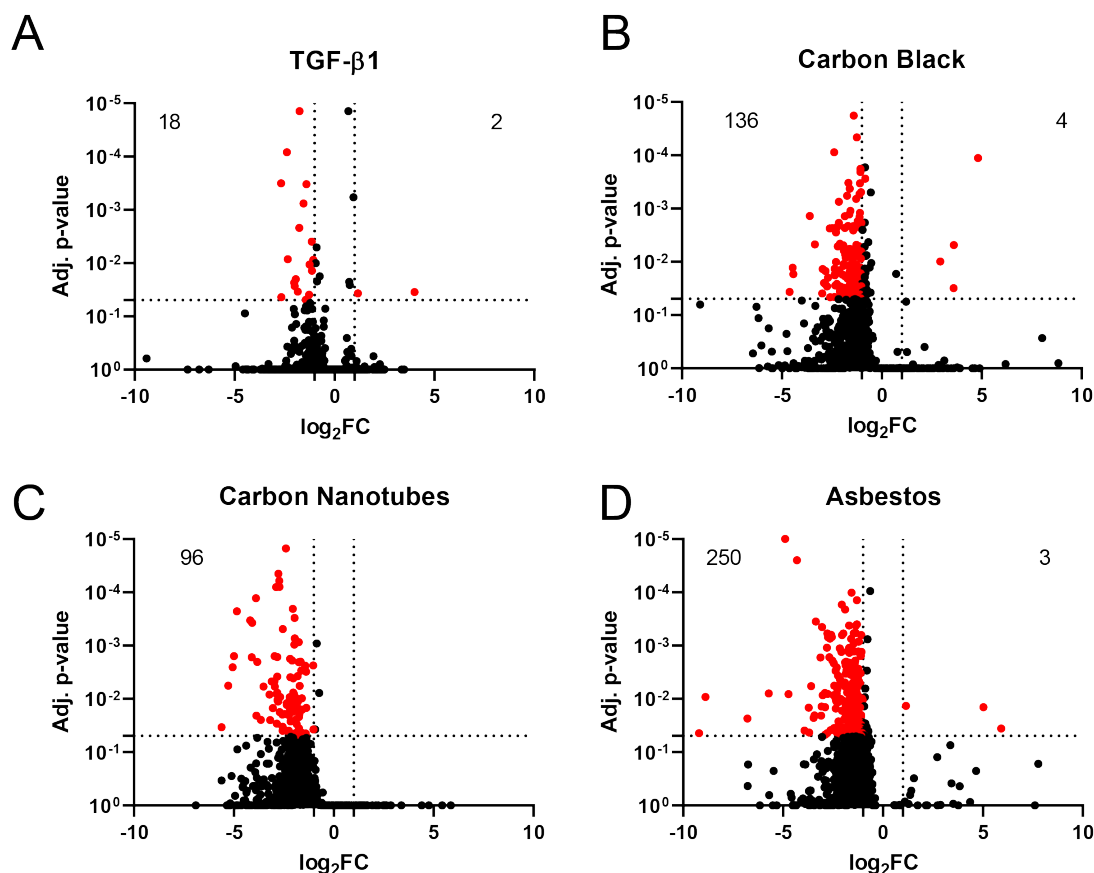


Figure 2.29: Volcano plots indicate significant fold changes associated with TGF- β 1 or nanomaterial exposure after 72 hours. (A) TGF- β 1 exposure results in significant increases (upper right) and decreases (upper left) in protein abundance. (B) Carbon black, (C) carbon nanotube, (D) and asbestos exposures result in a substantial down-regulation and minimal up-regulation of fibroblast proteins (red). Counts corresponding to changes in protein abundance are listed on each plot. Dotted lines indicate significance on the y-axis at $p < 0.05$ and on the x-axis at fold change > 2 or fold change < 0.5 .

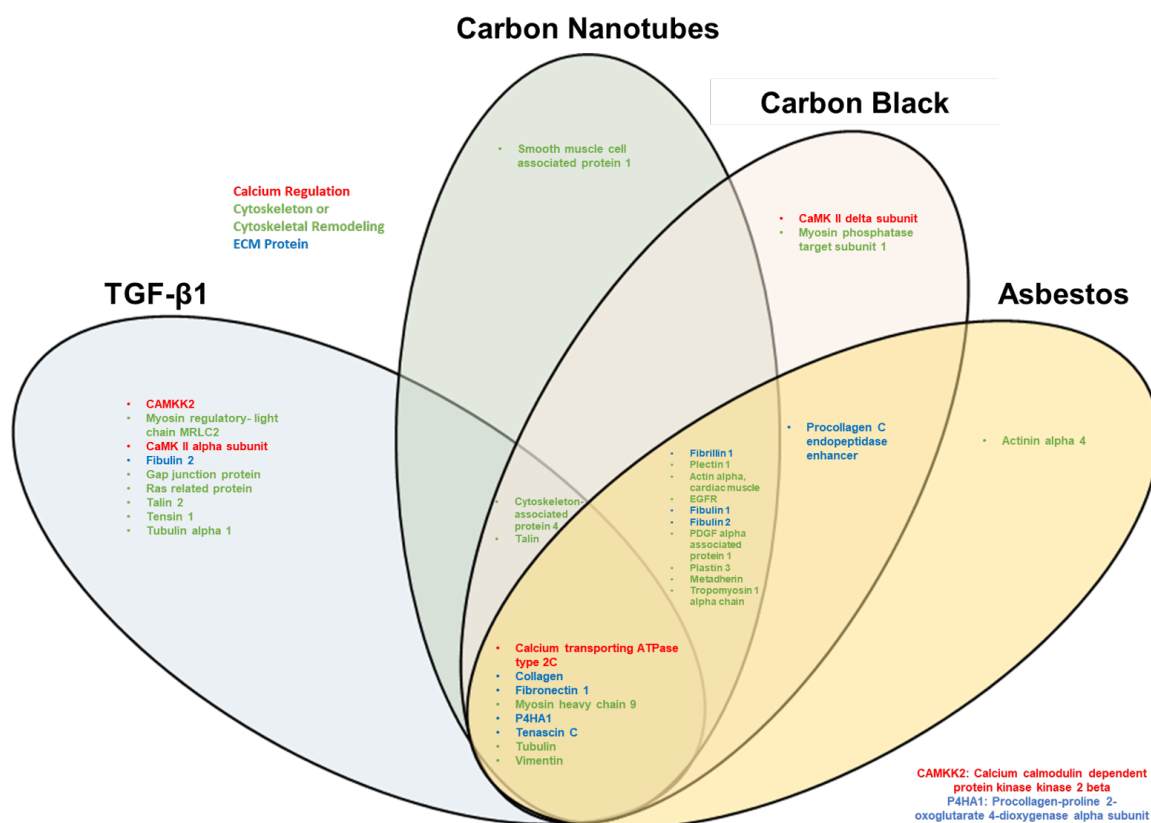


Figure 2.30: Protein Abundance Analysis: Top 50 Proteins. Proteomic analysis performed on IMR-90 fibroblasts exposed to recombinant TGF- β 1, carbon black, carbon nanotubes, and asbestos identified several significant protein changes after 72 hours. A selection of the top 50 significant proteins with fold changes >2 or fold change <2 compared to untreated conditions fall within three functional groups: cytoskeletal, calcium-regulating, and extracellular matrix proteins. Smooth muscle cell-associated protein (ACTA2), also known as alpha-smooth muscle actin (α -SMA), exhibits a significant increase after carbon nanotube exposure and is indicative of fibroblast to myofibroblast differentiation.

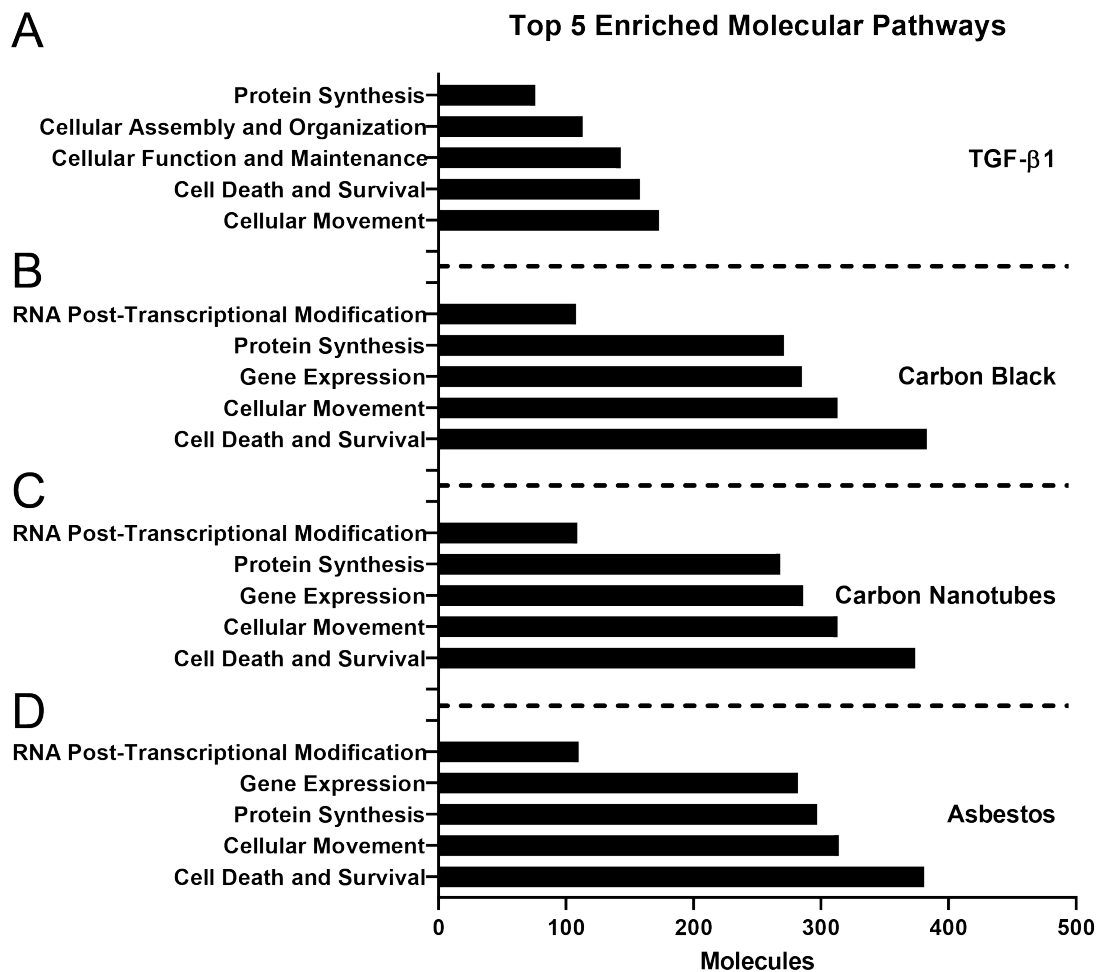


Figure 2.31: Protein Ontology: Significantly Enriched Molecular Pathways
Top five significantly enriched molecular pathways after 72 hour exposures to (A) TGF- β 1, (B) carbon black, (C) carbon nanotubes, (D) and asbestos. Changes in single proteins involved in the regulation of a few matching molecular pathways yield almost identical protein ontology profiles differing in the number of significantly up- or down-regulated proteins. Analysis was performed using Ingenuity Pathway Analysis.

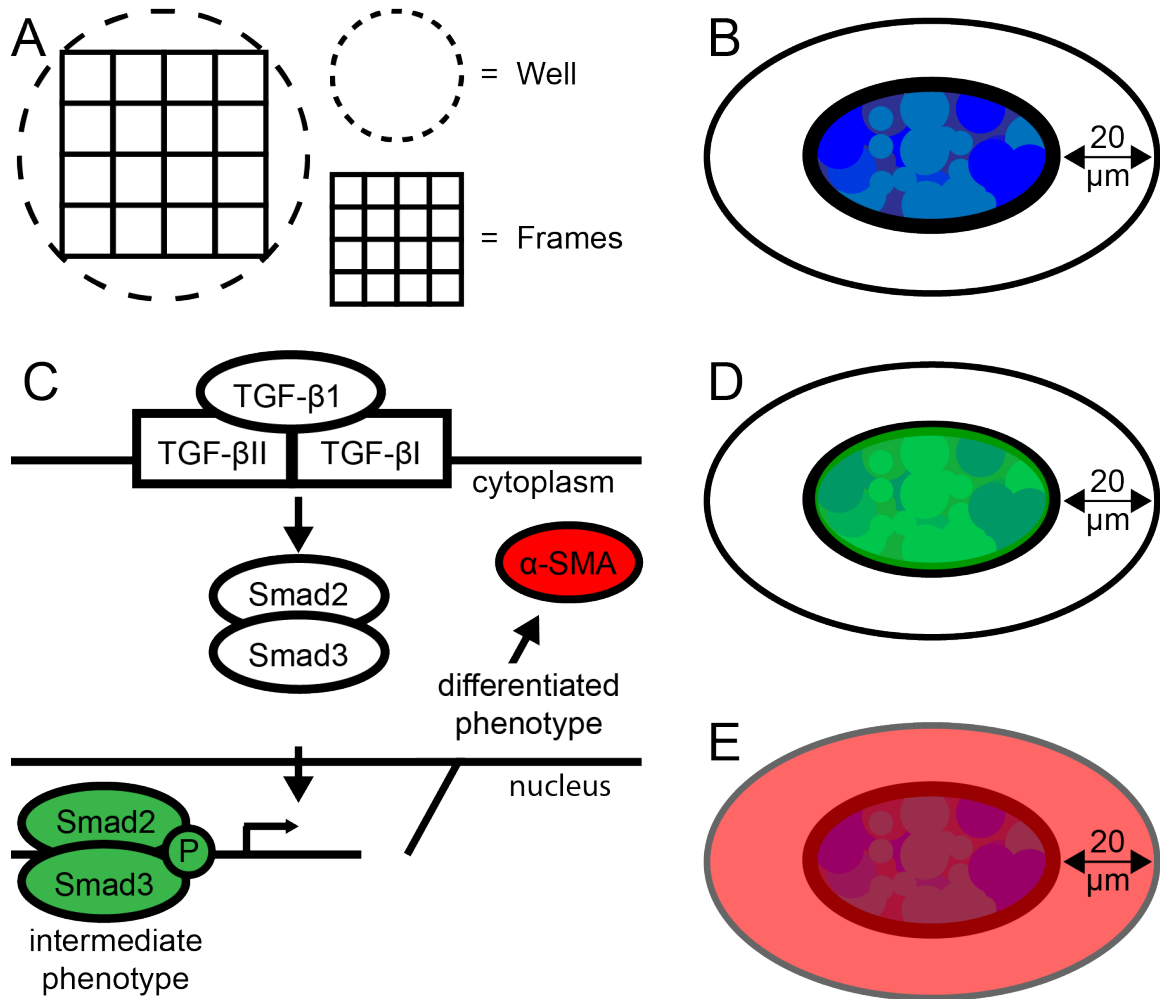
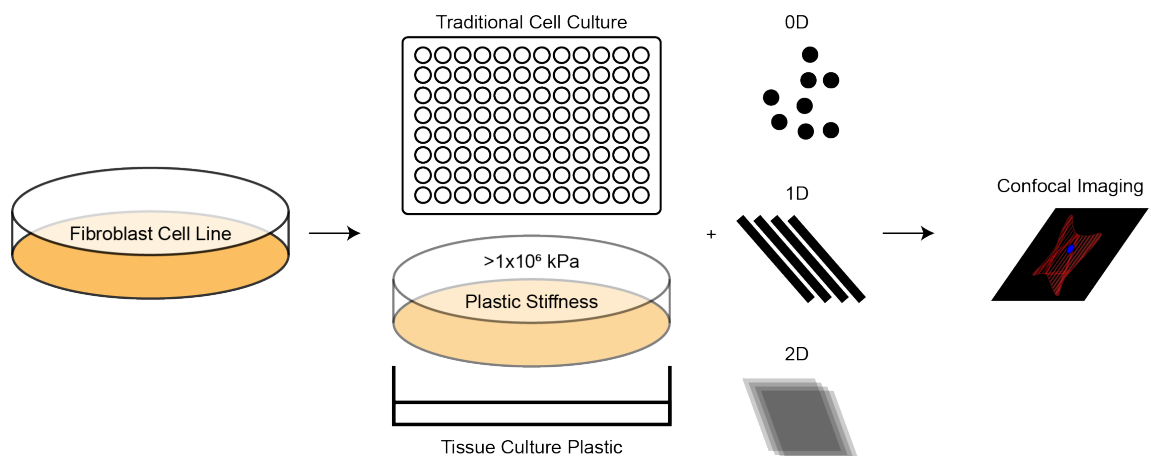


Figure 2.32: Confocal Global Image Stitching and Biomarker Profile Feature Extraction

(A) Confocal images were collected as individual frames and stitched together. (B) Confocal images of the nuclear channel were segmented to generate nuclear and perinuclear regions. (C) p-Smad2/3 and α -SMA TGF- β signaling pathway biomarkers indicating fibroblast to myofibroblast differentiation were evaluated in (D) nuclear and (E) perinuclear regions, respectively.

A Plating and Treatment Paradigm: Rigid Substrates



B Particle Exposure and Biomarker Immunofluorescence Imaging

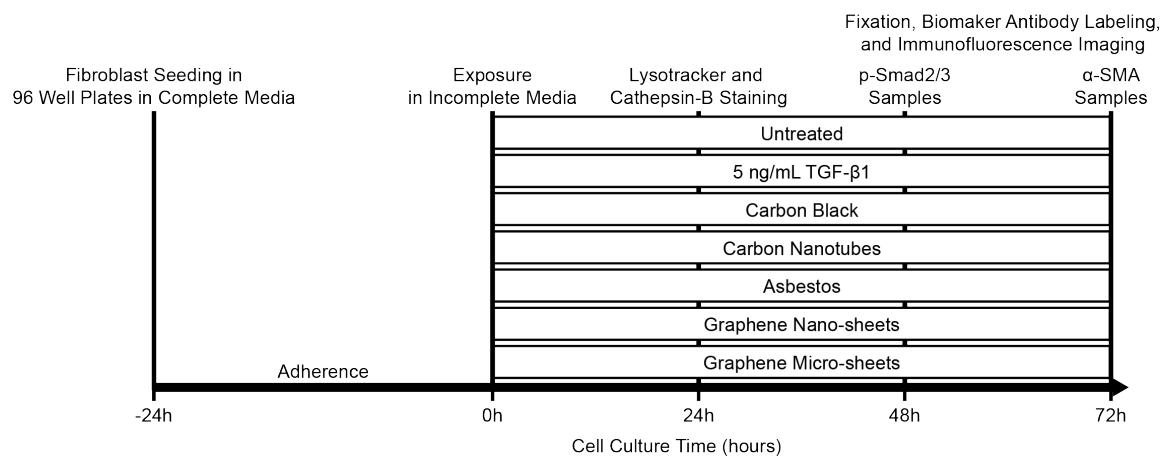


Figure 2.33: Experimental Plating Paradigm and Timeline: Nanomaterial Exposure on Rigid Substrates

(A) Experimental Plating Paradigm. (B) Experimental timeline for experiments on rigid substrates including lysosomal labeling and immunofluorescence imaging of TGF-β pathway biomarkers.

Viability Exposure Paradigm for Rigid and Soft Substrates

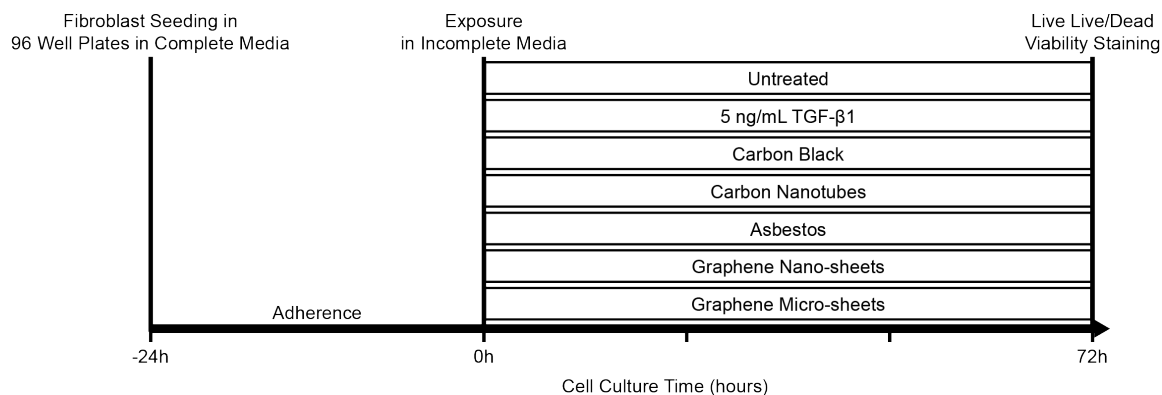


Figure 2.34: Experimental Timeline: Rigid and Soft Substrate Viability

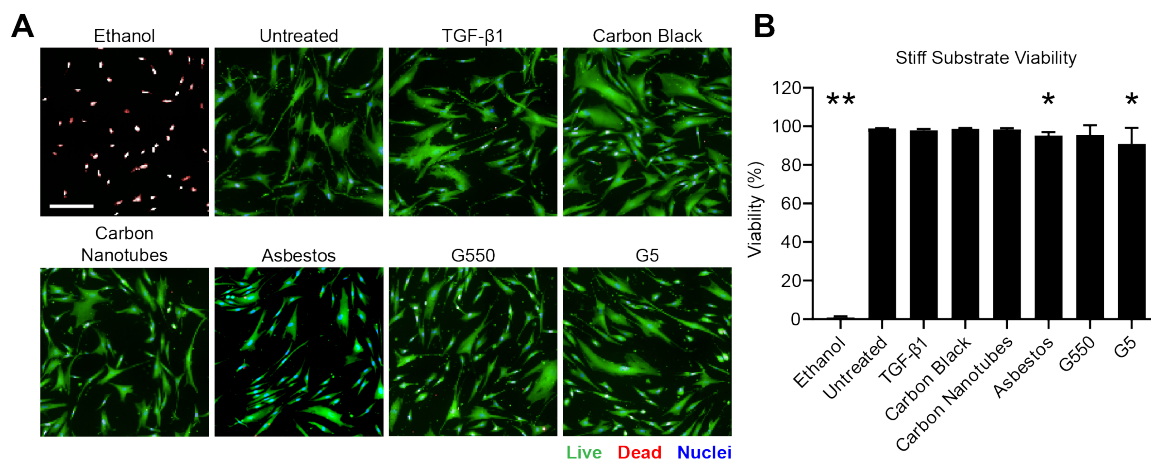


Figure 2.35: Fibroblast populations are viable on rigid substrates.

(A) Representative images of fibroblast populations 72 hours after exposure to all experimental conditions and an ethanol control. Calcein AM (green) labels live fibroblasts while Ethidium Homodimer-1 (red) stains porous fibroblast nuclei (Hoechst-stained, blue). Scale bar = 100 μ m. (B) Quantification of live cell populations 72 hours after exposure to all experimental conditions. Statistics were calculated using a Kruskal-Wallis test with a Dunn's post test. * p <0.05, ** p <0.01.

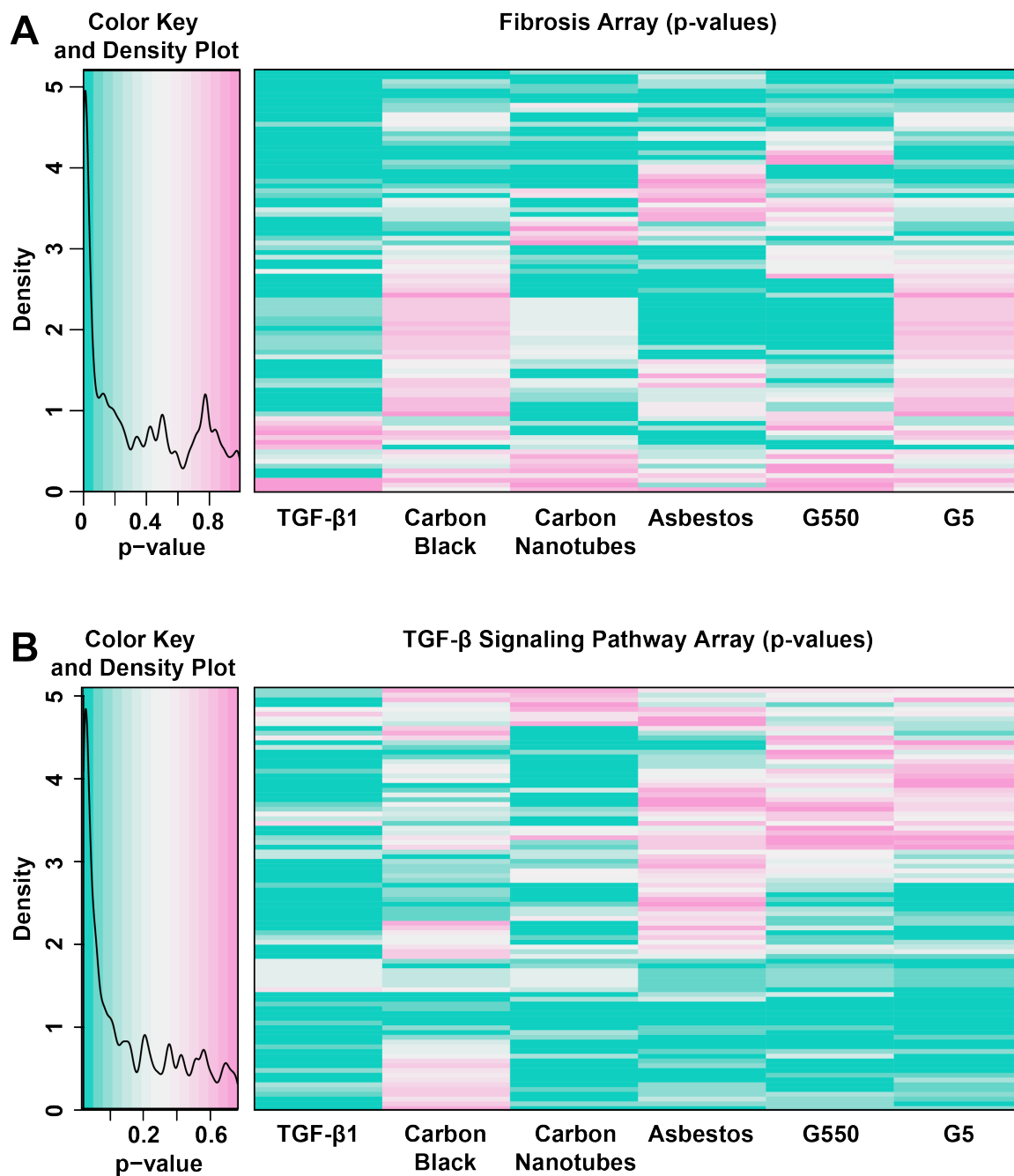


Figure 2.36: Significant Changes to Gene Expression After Nanomaterial Exposure on Rigid Substrates

Gene expression changes after 48 hours were evaluated by targeted (A) fibrotic and (B) TGF- β pathway gene expression arrays (Qiagen) against untreated samples. Matching sections of significant gene expression changes can be observed in each array profile. The TGF- β array identifies several groups of genes that are significantly changed after 5 ng/mL TGF- β and 0.6 $\mu\text{g}/\text{cm}^2$ nanomaterial exposures, but not after 0.6 $\mu\text{g}/\text{cm}^2$ carbon black exposure.

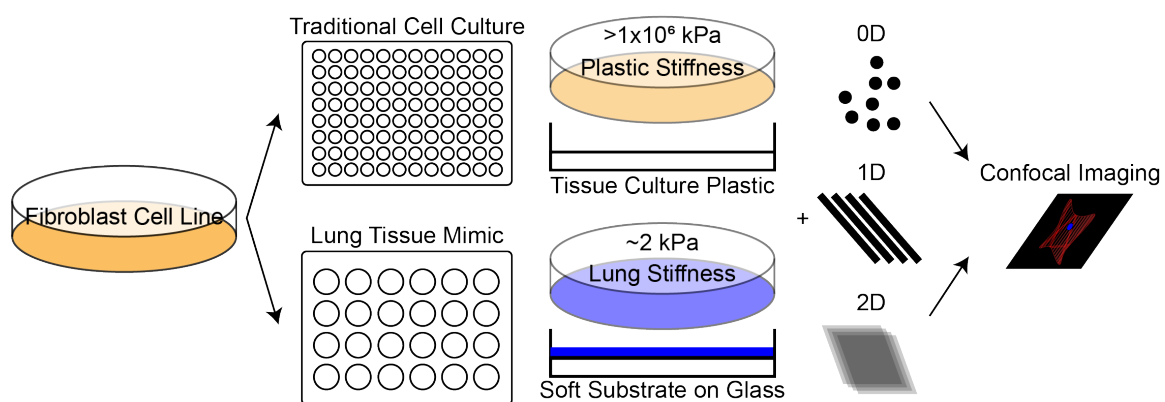


Figure 2.37: Incorporation of 2 kPa Soft Substrate into Plating and Treatment Paradigm.

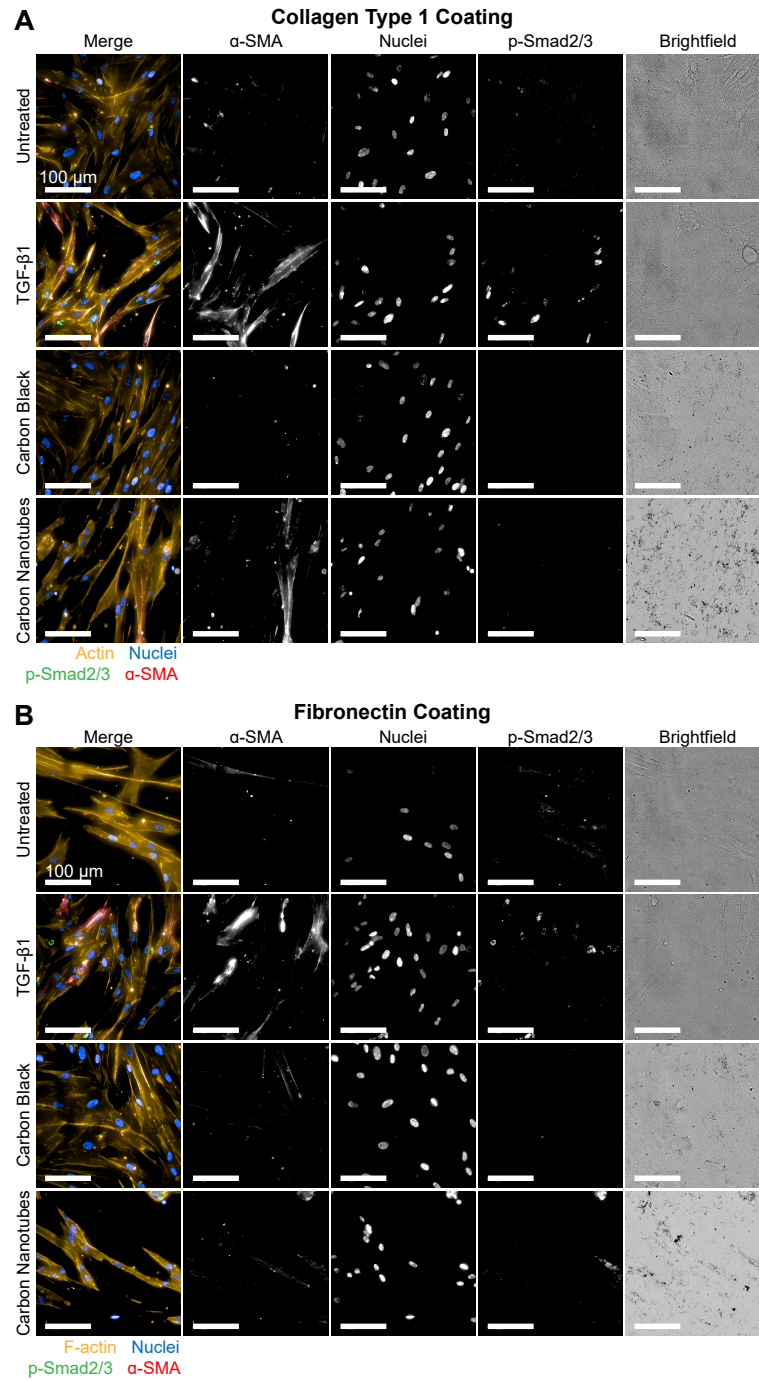


Figure 2.38: Collagen and Fibronectin Polyacrylamide Gel Coatings
IMR-90 fibroblast biomarker phenotypes are similar between (A) collagen 1-coated and (B) fibronectin-coated 2 kPa polyacrylamide gels across untreated, TGF- β 1, carbon black, and carbon nanotube conditions.

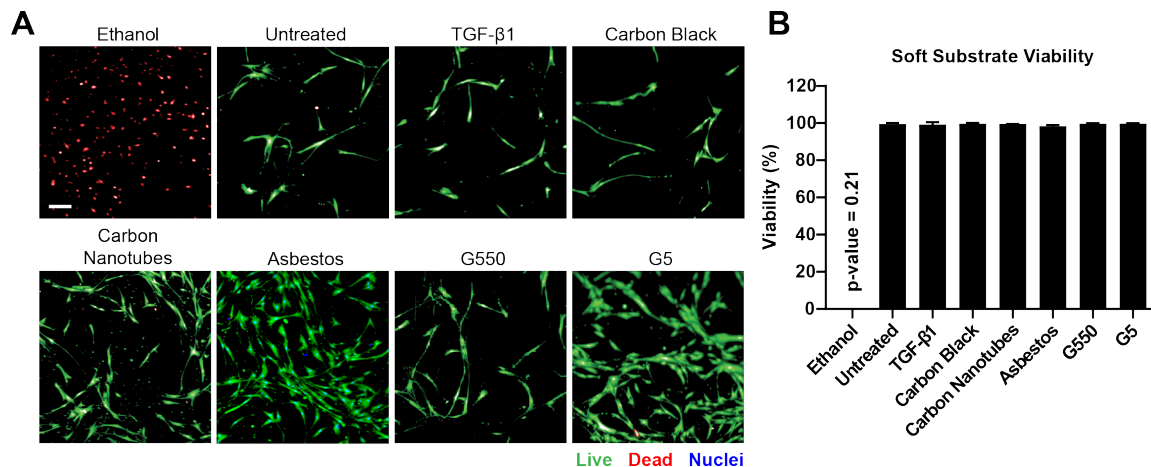


Figure 2.39: Fibroblast Viability on Soft Substrates.

Representative images of fibroblast populations 72 hours after exposure to all experimental conditions and an ethanol control. Calcein AM (green) labels live fibroblasts while Ethidium Homodimer-1 (red) stains porous fibroblast nuclei (Hoechst-stained, blue). Scale bar = 100 μ m. (B) Quantification of live cell population 72 hours after exposure to all experimental conditions. Statistics were calculated using a Kruskal-Wallis test with a Dunn's post test.

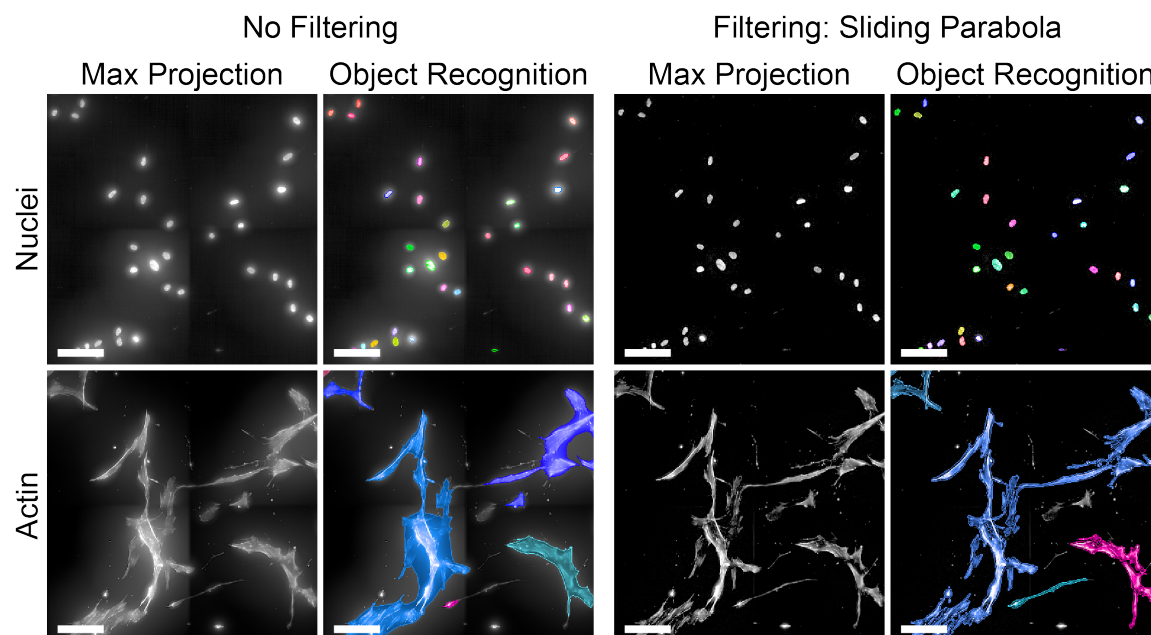


Figure 2.40: Sliding parabola filters improve segmentation on soft substrates.

Representative images with and without a sliding parabola filter on soft substrates for nuclear and actin channels. Image segmentation improves for both nuclear and actin targets (colored outlines) despite flattening 30-layer Z-stacks. Scale bar = 100 μ m.

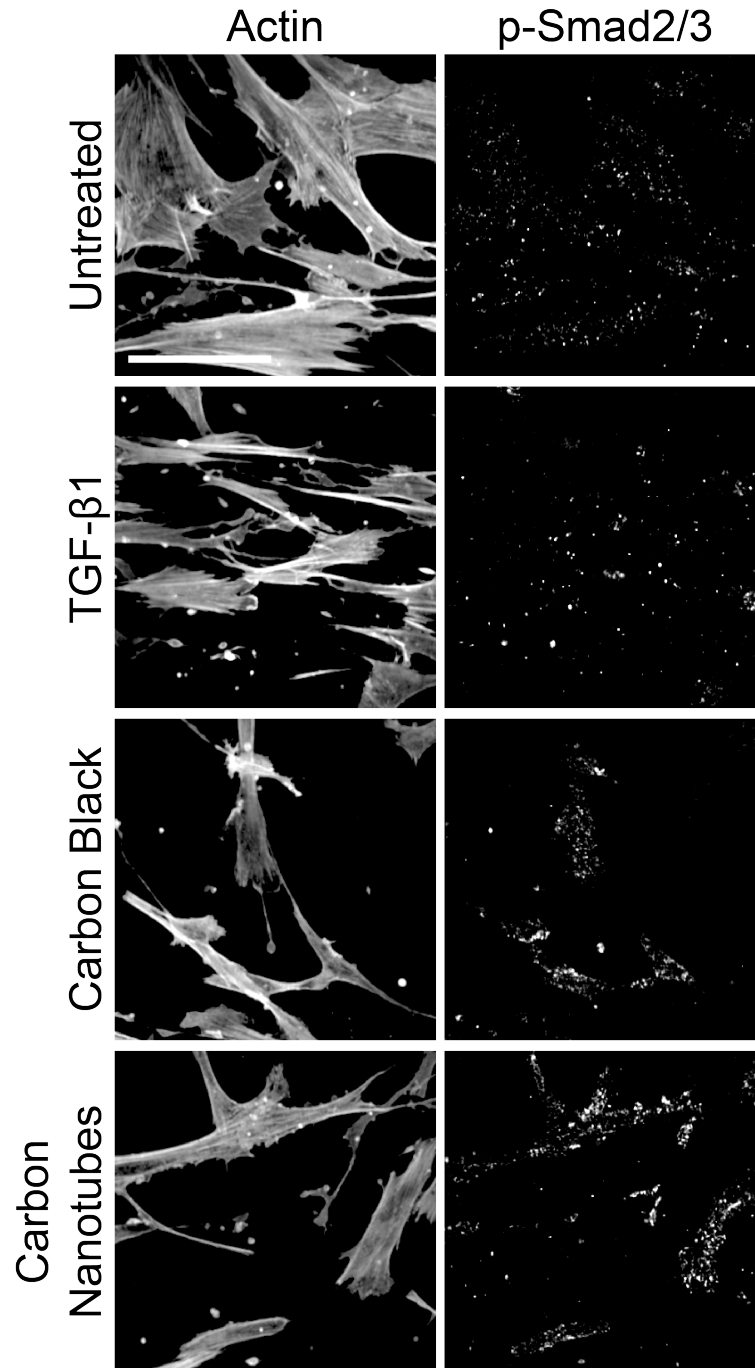


Figure 2.41: Fibroblast culture on soft substrates increases cytoplasmic Smad2/3 complex across all conditions.

Representative, enlarged images of observed p-Smad2/3 complex formation under untreated and nanomaterial conditions on soft substrates.

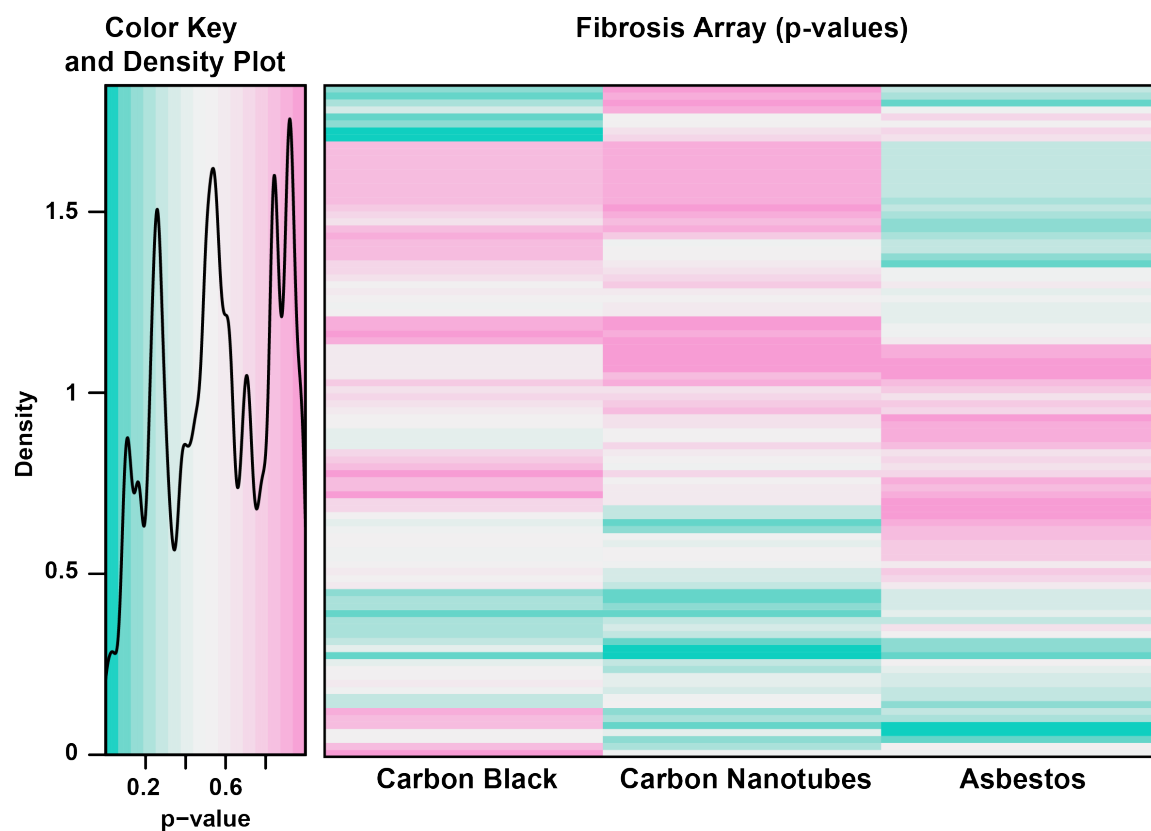


Figure 2.42: $2.86 \mu\text{g}/\text{cm}^2$ nanomaterial exposures exhibit fewer changes in fibrotic gene expression after 48 hours.

Gene expression changes after 48 hours were evaluated by a targeted fibrotic gene expression array (Qiagen) against matched untreated samples. Matching sections of significant gene expression changes are observed between carbon nanotube and asbestos exposures, but not after carbon black exposure. 48 hour $5 \mu\text{g}/\text{cm}^2$ nanomaterial exposures yield fewer significant gene expression changes compared to $0.6 \mu\text{g}/\text{cm}^2$ nanomaterial exposures.

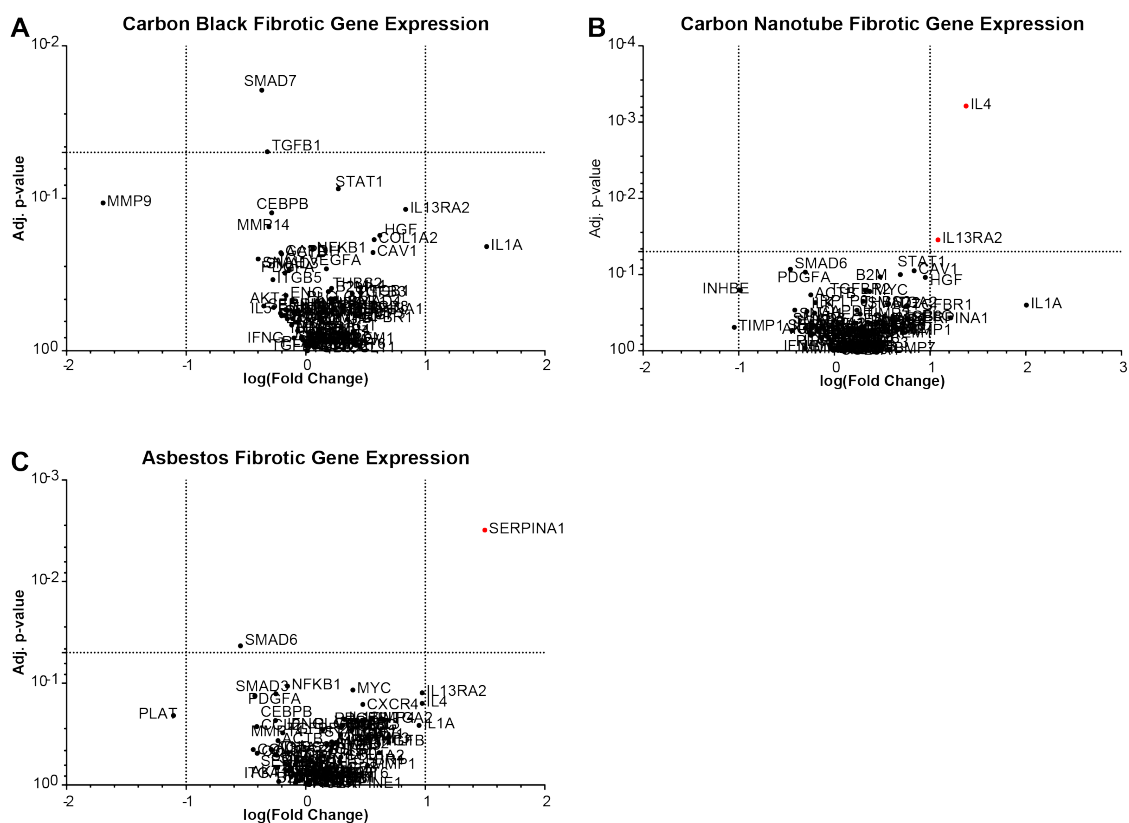


Figure 2.43: Volcano plots indicate significant fold changes after 2.86 $\mu\text{g}/\text{cm}^2$ nanomaterial exposures.

(A) 48 hour, 2.86 $\mu\text{g}/\text{cm}^2$ nanomaterial exposures yield fewer significant gene expression changes compared to 0.6 $\mu\text{g}/\text{cm}^2$ nanomaterial exposures. Dotted lines indicate significance on the y-axis at $p < 0.05$ and on the x-axis at $\text{fold change} > 2$ or $\text{fold change} < 0.5$.

Chapter 3

Developing 3D In Vitro

Nanoparticle Screening Tools to

Evaluate Drivers of Pulmonary

Fibrosis

Alysha Simmons, Norma Messier, Charles Vaslet, Ph.D., Ian Wong, Ph.D., Agnes Kane, M.D., Ph.D.

3.1 Abstract

Multicellular organization in 3D platforms has been shown to better recapitulate human physiology and gene expression when compared to traditional 2D cell culture on artificial substrates. 3D scaffold-free microtissues as models of facets of pulmonary

fibrosis present alternative high throughput and cost-effective nanomaterial screening tools for use over current in vivo fibrosis models. Four microtissue models were characterized by microtissue organization and molecular biomarkers as indicators of fibrotic activity. First, an IMR-90 fibroblast microtissue exhibited reduced viability before 72 hours, making them poor candidates for even acute toxicity testing. Second, preliminary research into the incorporation of collagen type 1 into IMR-90 fibroblast microtissues showed increased viability and compaction but resulted in decreased RNA and protein yields. Third, a microtissue model of IMR-90 fibroblast and BEAS-2B epithelial cell interactions was developed to interrogate the fibroblast-epithelial signaling axis in the context of nanomaterial-driven fibrosis. Preliminary research showed that microtissues were viable long-term in the absence of recombinant TGF- β 1 exposure, capable of depositing ECM, and predominantly comprised of myofibroblasts and fibroblast-like epithelial cells, but were resilient to high concentrations of nanomaterial exposure as measured by TGF- β 1 secretion. Finally, a microtissue model of immortalized primary fibroblast and THP-1 macrophage interactions was developed to interrogate the stability of cadherin-11-mediated physical cell-cell interactions in the presence of nanomaterials and the resulting regulation of TGF- β 1 secretion. Overall, 3D microtissue modeling specific cell-cell relationships as facets of fibrosis offer insight into the drivers of the complex fibrotic systems observed in in vivo models and could be developed into sensitive screening tools that are cost-effective and readily available for use in academic and regulatory settings.

3.2 Introduction

3.2.1 2D vs. 3D In Vitro Models Utility

In vivo models model the systemic response to a toxicant or drug but remain costly, time-consuming, and are sometimes translationally irrelevant. Scientists have suc-

cessfully recapitulated features of several organ systems by integrating individual or multiple technologies into in vitro toxicant and drug screening tools, thus increasing their human physiological and molecular translational relevance and decreasing time and costs [1]. As a result, 3D models experienced a publication boom in the 1990s, increasing to over 1400 publications by 2020. In that time, 3D models have evolved to include bioengineered technologies consisting of biocompatible and biomimetic substrates and dynamic plug-and-play technologies that allow for the integration of physiologically important characteristics into the previously untenable complex in vitro models. These technologies include but are not limited to, synthetic ECM proteins, mechanically tunable substrates, scaffold-based and scaffold-free 3D cell culture platforms, 3D bio-printed organotypic tissues, and plug-and-play organ-on-a-chip or microfluidic systems [1, 2]. While each platform must be carefully tuned to the experimental question, several models of pulmonary fibrosis already exist and ask critical questions about acute versus chronic responses, cell-type-specific responses, and how best to recapitulate routes of exposure and drug treatment. Sacchi et al. and Faber et al. astutely pose several technical and scientific questions to this end about the current and future utility of these platforms in fibrosis research. Five key questions form some of the basis for this thesis work. 1) What model type and level of complexity is required to model fibrosis? 2) Which biomarkers best evaluate fibrotic endpoints in each model? 3) Which models can stage fibrosis or recapitulate the stages of fibrosis? 4) Are induced pluripotent stem cells (iPSCs) or patient-derived cells necessary to evaluate fibrotic endpoints? 5) Are fibrosis models improved by the integration of a vascular network, the use of different cell culture media supplements, or the inclusion of a clearance mechanism [3, 4]? This thesis work aims to address some of these questions using some of the technological advances described herein.

3.2.2 Classic Rodent Models of Fibrosis

Rodent models are valuable for their ability to test toxicants in whole, living systems for up to two years compared to less complex, acute, mono- or multicellular in vitro cell culture models. Until recently, in vitro models only evaluated one or two 2D cell-type interactions, were incapable of testing different routes of toxicant administration, quantified dose-dependent systemic effects, and studied physiological organization. As a result, several rodent models evaluating the fibrogenic response after chemical or particle exposure have been developed and continue to be utilized as control conditions in more complex exposure research scenarios. For example, bleomycin, a cancer drug used to induce fibrosis-like lung damage in rodent models, causes inflammation, significant increases in TGF- β 1 secretion and TGF- β pathway signaling, and fibronectin and collagen deposition as early as seven days and fibrosis-like characteristics by as soon as nine days after exposure [5-7]. Paraquat, an herbicide and potent fibrogenic agent, causes oxidative stress and redox cycling in alveolar cells [8-12]. Finally, crystalline silica and crocidolite asbestos are fibrogenic and carcinogenic model particles that illustrate the effects of decreased clearance, inflammation, reactive oxygen species production, and granuloma formation with differences in response to small versus aggregated particle clusters [13-24]. These models can be lengthy and time-consuming but have informed in vitro models of increasing complexity that ask similar questions.

3.2.3 Modeling Fibrosis Using 3D Spheroids

Several previously characterized cell types have already been incorporated into bio-engineered model systems including fibroblasts, epithelial cells, endothelial cells, and white blood cells to generate primary cell organoids, induced pluripotent stem cell organoids, cell line-derived spheroids, bio-fabricated or bio-printed microtissues or microenvironments, and vascularized microfluidic chip platforms [3, 4, 25]. 3D hy-

drogels function as biocompatible scaffolds that incorporate microenvironmental cues, including integrin binding sites, fiber density and strength, pore size, and potential for matrix degradability. Hydrogel and ECM components can include reconstituted collagen types, Matrigel, gelatin, reconstituted fibrin, reconstituted elastin, hyaluronan, alginate, purified glycosaminoglycans, nanocellulose, silk fibroin, and many other types of proteins [26]. 3D models embedded within ECM scaffolds allow for the evaluation of secreted soluble factor diffusion, directed proteolysis and scaffold remodeling, the formation of chemotactic and mechanical gradients, cell invasion phenotypes, and phenotypic plasticity, all of which were previously evaluated in vivo [26]. Furthermore, 3D models offer the ability to study lung cell structure and function based on cell sorting, assess acute and chronic endpoints, and evaluate repeated exposure endpoints and adaptation in a high throughput capacity [27]. These endpoints are complicated to assess in in vivo models but are becoming more accessible with increased bioengineering and 3D in vitro modeling capabilities.

Additionally, infinitely stiff substrates drive the unnatural organization of the cell cytoskeleton, mature focal adhesion formation, and polarization that does not reflect in vivo conditions. 3D cell cultures experience cell-cell and cell-matrix interactions that can circumvent the effects of confluence present in 2D cell culture and prevent abnormal cell-culture substrate-driven behavior. However, many 3D cell cultures still lack essential, complex in vivo features, including flow and perfusion, integration of clearance mechanisms, and accurate representations of in vivo physiological organization. However, merely comparing 2D, 3D, and in vivo model complexity does not invalidate their translational capabilities and requires physiologically-relevant benchmarks garnered from case studies or in vivo rodent models to assess true model potential. Within 3D models, cells exhibit gene expression, polarization, and cell-cell interactions that better mimic in vivo conditions and can be used as benchmarks of model utility. Finally, 3D cell culture allows for the evaluation of ECM interac-

tion, remodeling, and deposition in ways that are not possible using traditional 2D cell culture methods. With the increasing 3D model utility, discussion surrounding important benchmarks of model functionality suggests that models should be evaluated for physiological organization and morphology, the presence of cell-cell junctions, proliferative rate and apoptosis, gene expression and evaluation by other omics platforms, in addition to showing efficacy to decrease time and costs associated with high-throughput screening relative to in vivo models [28]. For this thesis research, benchmark objectives for fibrosis model utility focused on physiological organization, fibrotic gene expression arrays, and TGF- β family ligand secretion.

Non-adherent Scaffold-free Microtissues

Platforms without integrated ECM or synthetic coatings to promote cell adherence allow for scaffold-free growth. Non-adherent platforms in which cell-cell interaction drives spontaneous spheroid self-assembly presents opportunities to study cell-cell interactions, ECM deposition, viability, omics analysis, and imaging analysis of one or more cell types in three-dimensions [29, 30]. Non-adherent 3D models include hanging-drop, non-adherent hydrogels, and some types of microfluidic devices. While hanging-drop and microfluidic device platforms have several technical difficulties associated with their maintenance and sample collection, the use of printed or molded non-adherent hydrogels is valued for their cost-effective, high-throughput, and reproducible features.

3D in vitro models can now interrogate the relationship between nascent ECM, phenotypic plasticity, and fibrotic potential by assessing the dysregulation of connective tissue deposition or failure to re-epithelialize regions of damage and trace these endpoints to a disorder of signaling between epithelial cells and fibroblasts [31, 32]. Endpoints indicating these aberrant cell-cell interactions in 3D model systems can be assessed by evaluating epithelial cell barrier formation and permeability, cell

viability, fibroblast or epithelial cell proliferation, fibroblast to myofibroblast differentiation, epithelial to mesenchymal differentiation, and identification of regions of nascent ECM deposition or remodeling, among other fibrosis benchmarks [32, 33].

Modeling the relationship between human lung cell types in a non-adherent 3D microenvironment offers the opportunity to evaluate rare or transient cell relationships, an absent feature of *in vivo* models. Initially, the third aim of this thesis was to develop a 3D *in vitro* model of a fibroblastic focus; however, during the evolution of this research, we developed four models in an attempt to address shortcomings associated with a fibroblast-only spheroid model and evaluated more complex relationships between fibroblasts, epithelial cells, and macrophages in the context of fibrosis. Non-adherent hydrogels allow for single-cell suspensions to spontaneously form clusters or microtissues comprised of one or multiple cell types in an unimpeded and undirected fashion, making them ideal for studying multicellular interactions over time. Cell sorting in non-adherent hydrogels is thought to be driven by junctional proteins and cytoskeletal organization and can be destroyed by the introduction of a substrate with high stiffness, meaning that specific cell-cell interactions can only be observed in 3D models embedded in soft hydrogels or in non-adherent platforms, which we posit is necessary to evaluate interactions within the epithelial-fibroblast signaling axis [34-38] and physical interactions between fibroblasts and macrophages [39].

3.2.4 Existing 3D Fibroblast Microtissue Culture

3D fibroblast spheroids, as models of fibroblastic foci, the air-liquid interface, and cancer-associated fibroblast function, have been used to evaluate sustained fibroblast quiescence, fibroblast-epithelial cell crosstalk, and tumor growth endpoints, respectively. WI-38 fibroblasts, a similar cell line to IMR-90 fibroblasts, and are commonly utilized in both 2D and 3D *in vitro* models. Carbon nanotube and crystalline silica exposures induce profibrotic gene expression, activate the TGF- β signaling pathway,

and triggered fibroblast to myofibroblast differentiation in WI-38 fibroblasts embedded in a collagen matrix [40]. Zeinali et al. successfully modeled and inhibited microvasculature formation in fibroblast and endothelial cell cocultures exposed to the anti-fibrotic drug Nintedanib [41]. Research into drivers of tumor survival and tumor growth shows that tumor growth was enhanced by soluble factors secreted by fibroblasts in monolayer but not by 3D fibroblast spheroids.

Additionally, fibroblast spheroids compacted over 96 hours and did not exhibit any proliferation, which we also observed in our primary fibroblast microtissue model [42]. Finally, 3D models of human bronchiole have been developed using tissue engineering techniques. Bioreactors provide sources of stimulation that mimic bronchiole expansion, a mechanical stimulus, an air-liquid interface component, and a perfusion component applied to connective tissue regions with adherent epithelial cells and fibroblast [43]. Fibroblasts grown in bioreactors show increased response to estrogens in addition to decreased cytoskeletal, adhesion, migration, cell cycle, and TGF- β signaling pathway gene expression [44]. This thesis work aimed to exploit the success of these models to evaluate site-specific differentiation driven by toxic particle exposures in stable fibroblast microtissues as a model of an early fibroblastic focus.

3.2.5 3D Fibroblast and Epithelial Cell Microtissue Cocultures

The epithelial-fibroblast signaling axis is defined as the many physical and chemical interactions between the two cell types to regulate homeostasis, the phases of wound healing, and pathological responses. In vitro and in vivo research posits that the dysregulated interplay between these two cell types is responsible for fibrogenesis. IMR-90 fibroblasts mixed with human bronchial epithelial cells in collagen gels spontaneously form multilayer, bronchiole-like organization [43]. Soluble factors in fibroblast conditioned media increase epithelial cell surfactant production [45-51], and

physical fibroblast-epithelial cell interactions through gap junctions drive cell growth secondarily mediated by the surrounding ECM [45-51]. Endpoints to benchmark 3D fibrosis platforms can include fibroblast growth factor, insulin-like growth factor, interleukin factors, plasminogen activator inhibitor-1, and TGF- β signaling factors in epithelial cells and fibroblasts [52]. In a bronchiole model of BEAS-2B bronchial epithelial cell and WI-38 fibroblast coculture was compared between ECM-coated rigid substrates and Matrigel embedded models. Rigid substrate cell culture increases cell proliferation, but only collagen coatings significantly increase TGF- β 1 expression, while fibronectin produces network-like clusters of fibroblasts.

Alternatively, 3D culture in Matrigel forms network-like alveolar structures with cuboidal epithelial cells and spindly fibroblast morphologies [53]. Several fibroblast and epithelial cell 3D coculture models have recently been developed to evaluate cigarette and electronic cigarette toxicity. Organotypic microtissues, grown at the air-liquid interface, found that smoke extract induced changes in cell proliferation, cell stress, inflammatory, and apoptotic gene pathways with fewer overall changes observed in cocultures than in separate monocultures, suggesting that coculture promotes stability in the transcriptomic profiles and decreases overall stress [54, 55]. Alternative approaches to benchmarking model utility have included comparing 2D monoculture samples to established, commercially available 3D platforms, in addition to toxicity testing using or developing a tiered testing approach. Tiered testing refers to toxicant categorization based on physicochemical or toxicity characteristics that prioritize the order of toxicity testing. Using several well-characterized toxicants with known mechanisms of action, A549 epithelial cell and 3T3 fibroblast 2D monoculture platforms were compared to two, 3D air-liquid interface platforms to demonstrate the utility of LDH production, viability, and metabolic activity endpoints to generate a tiered testing paradigm for toxic chemical and ultrafine particles and emphasizing increased sensitivity in 3D model systems [56]. This type of experimental comparison

highlights discrepancies in cell phenotype associated with 2D and 3D model systems. We hypothesized that our 3D, scaffold-free microtissue models would be more sensitive to toxic particle exposures after the removal of confounding synthetic substrate interactions and multicellular interactions.

3.2.6 3D Fibroblast and Macrophage Microtissue Cocultures

In vivo alveolar and recruited macrophages secrete inflammatory cytokines, MMP-9, plasminogen activator, TGF- β 1, and increase TGF- β pathway signaling to drive inflammation during fibrogenesis [3]. Furthermore, two, 2D models have shown that cadherin-11-driven physical interactions between fibroblasts and macrophages mediate TGF- β 1 secretion and fibroblast to myofibroblast differentiation [39, 57]. This research inspired us to recreate these interactions in a 3D non-adherent system to evaluate this physical interaction in the absence of a cell culture substrate. Moreover, we aimed to assess if the preferential interaction of macrophages with deposited particle aggregates would disrupt or strengthen this physical interaction with concurrent TGF- β 1 secretion, or if particle-interaction would disrupt cadherin-11-mediated signaling and only signal through secreted TGF- β 1.

Very recent research in a 3D model of fibroblast and macrophage coculture in non-adhesive agarose hydrogels evaluated the impacts of cell count on fibrotic endpoints. Microtissues larger than 300 μ m experienced inflammasome activation, central hypoxia, decreased collagen deposition, and increased α -SMA and decreased MMP gene expression, with all microtissue sizes experiencing ongoing compaction out to 7 days [58].

3.2.7 Characterizing 3D Scaffold-Free Lung Microtissue Models

Allowing cells to experience biophysical and biochemical cues independent of synthetic substrates is essential to understand drivers of cell-cell interactions and multicellular organization and is necessary for determining the fewest number of mandatory components needed to recapitulate epithelial and stromal tissue compartments accurately. Furthermore, these advanced physiological structures suggest that there is an interplay between fibroblasts, epithelial cells, and macrophages that is critical for regulating cell phenotypes in both healthy and fibrotic 3D lung microenvironments [39, 59-62]. Previously published research from our lab proposes the use of a triculture model to monitor the secretion of profibrotic mediators, changes in gene expression, and regulation of ECM deposition and remodeling out to 7 days. Additionally, the triculture model was benchmarked to known in vivo endpoints to show recapitulation of profibrotic gene expression after exposure to pristine, toxic carbon nanoparticles [22]. Future experimental endpoints indicating nanomaterial toxicity can be assessed using 3D non-adherent model systems by quantifying cell viability, fibroblast or epithelial cell proliferation, fibroblast to myofibroblast differentiation, epithelial to mesenchymal differentiation, markers of macrophage activation, and identification of regions of nascent ECM deposition or remodeling at acute time points. As a third aim, we deconstruct the triculture model and develop and characterize fibroblast, epithelial cell, and macrophage cell-cell interactions in the context of pulmonary fibrosis using non-adherent in vitro microtissue models. Finally, using a panel of well-characterized nanomaterials, microtissue morphometry, and TGF- β signaling endpoints, we benchmark each model to known fibrogenic endpoints based on known human and in vivo exposure data.

3.3 Materials and Methods

3.3.1 Cell Culture

BEAS-2B lung bronchial epithelial cells and THP-1 human monocytic cells were selected because they have been widely used for nanotoxicology assays.

BEAS-2B (ATCC CRL-9609) were cultured in BEGM media with SingleQuots supplement kit (Lonza CC-3170) at 37°C in 5% CO₂. BEAS-2B cells incubate in 0.25% trypsin until detached from the bottom of the flask. Cells are collected and centrifuged for 5 minutes at 1000 rpm at 4°C. Cells are re-seeded in collagen-coated flasks (Corning 354485) at a 1:10 split, maintained below 80% confluence, and only used in experiments below passage 30.

THP-1 human monocytic cells (THP-1, ATCC TIB-202) were cultured using RPMI-1640 (Gibco 11875093) with 10% heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals S12450) and 1% Penicillin-Streptomycin (P/S) (Thermo Fisher 15140122) at 37°C and 5% CO₂ in 100 mm low-attachment cell cultures dishes. Before use, THP-1 cells were differentiated into macrophages with 50 nM phorbol 12-myristate 13-acetate (PMA) (Fisher Scientific 5244001MG) for 72 hours.

IMR-90 (ATCC CCL-186) human, lung fibroblasts were cultured in cell culture treated flasks in Eagle's Minimum Essential Medium (EMEM) (ATCC 30-2003) with 15% fetal bovine serum (Atlanta Biologicals S12495H) and 1% P/S at 37°C in 5% CO₂. Fibroblasts were cultured to below 85% confluence before splitting and utilized until passage 15. Cells were collected and centrifuged for 5 minutes at 1000 rpm at 4°C.

Primary hTERT immortalized fibroblasts (ATCC CRL-4058) are cultured in fibroblast basal media (ATCC PCS-201-030), low-serum components (ATCC PCS-201-041), and 0.3 µg/mL puromycin (Gibco A1113803) at 37°C in 5% CO₂. Fibroblasts

were cultured to below 80% confluence before splitting and utilized until passage 20. Cells were collected and centrifuged for 6 minutes at 226 x g at 4°C.

All experiments were placed in an incubator with 5% CO₂ at 37°C with or without treatment with nanomaterials, 50 μ M SB431542 (TGF- β 1 inhibitor, R&D Systems 1614) or 5 ng/mL TGF- β 1 (R&D Systems 240-B-010) at seeding. If microtissues are fixed, they are rinsed twice in 1x PBS for 5 minutes each, and fixation occurs in 10% neutral buffered formalin.

3.3.2 Preparation of Nanomaterial Suspensions

Carbon black (M120), consisting of isometric elemental carbon nanoparticles, was purchased from Cabot Corp. (Boston, MA) and, as previously characterized, has a primary diameter of 75 nm and a total surface area of 35 m²/g. Multi-walled carbon nanotubes (MWCNT-7) from Mitsui and Co. Ltd. (Tsukuba Ibaraki, Japan) were generously provided by Dr. Gunter Oberdörster (University of Rochester, Rochester, NY). The 550 nm and 5 μ m graphene flakes were purchased from Cabot Corp. and Graphene Supermarket, respectively. The physiochemical descriptions of these materials are summarized in Table 2.2. All material stock suspensions were prepared using sterile methods under a Class IIB biological safety hood with external exhaust to ensure sterility and maintenance of the endotoxin-free environment. Nanomaterial stocks were suspended in sterile, endotoxin-free, 10% dipalmitoylphosphatidylcholine (DPPC, Avanti Polar Lipids 850355P) and 3% BSA (Sigma-Aldrich A9576) solutions. Stocks were water bath sonicated (Branson Ultrasonic Corporation, Danbury, CT) for 5 minutes and brought to 1000 μ g/mL concentrations in 1% bovine serum albumin-containing (BSA) (Sigma-Aldrich A9418) media and then water bath sonicated for an additional 45 minutes. Stocks were kept at 4°C and sonicated again before dilution to working stock concentrations in complete EMEM cell culture media. This dispersion technique produced uniform suspensions of individual particles and flakes (carbon

black and graphenes) or individual fibers and small agglomerates (carbon nanotubes) as described previously [20]. Endotoxin levels in the stock particle suspensions were determined using the gel-clot assay and were less than 0.31 EU/mL (Associates of Cape Cod, Inc.). 1000 $\mu\text{g/mL}$ stocks of nanomaterials are diluted in incomplete media (EMEM, 2.5% FBS, and 1% P/S) to 3.6 $\mu\text{g/mL}$ concentrations, equal to a seeded concentration of 0.6 $\mu\text{m/cm}^2$ on a T-150 flask.

3.3.3 Microtissue Formation

IMR-90 fibroblasts were seeded into 256-microwell hydrogels at 500 cells per spheroid. IMR-90 spheroids are grown in Eagle’s Minimum Essential Medium (EMEM) (ATCC 30-2003) with 15% fetal bovine serum (Atlanta Biologicals S12495H) and 1% P/S at 37°C in 5% CO₂. Collagen (rat tail collagen type 1) is added to IMR-90 fibroblast microtissues in 81-microwell hydrogels at seeding to a final concentration of 141 $\mu\text{g/mL}$ in complete Eagle’s Minimum Essential Medium (EMEM) (ATCC 30-2003) with 15% fetal bovine serum (Atlanta Biologicals S12495H) and 1% Penicillin-Streptomycin (Thermo Fisher 15140122) and kept at 37°C in 5% CO₂.

Primary fibroblast and THP-1 macrophage microtissues are seeded in a 3:1 ratio in 256-microwell hydrogels in fibroblast basal media (ATCC PCS-201-030), low-serum components (ATCC PCS-201-041), and 0.3 $\mu\text{g/mL}$ puromycin (GIBCO A1113803) and maintained at 37°C in 5% CO₂.

IMR-90 fibroblast and BEAS-2B epithelial cell microtissues are seeded in a 1:2 ratio, respectively. Microtissues are seeded in EMEM with added 15% FCS with SingleQuotes additives except for bovine pituitary extract (BPE) and human epidermal growth factor (hEGF). After 24 hours, microtissues are switched into incomplete maintenance media consisting of EMEM with 0.5% FCS with SingleQuotes additives except for bovine pituitary extract (BPE) and human epidermal growth fac-

tor (hEGF). (SingleQuotes refer to Hydrocortisone, Epinephrine, Transferrin, Insulin, Retinoic Acid, Triiodothyronine, Gentamicin/Amphotericin-B)

If exposed to nanomaterials, nanomaterials are mixed into cell suspensions prior to seeding. Microtissues are allowed to settle by gravity and spontaneously form around nanomaterials. 5 ng/mL recombinant TGF-B1 (RandD Systems 240-B), 1 μ g/mL recombinant MMP-3 (Abcam ab96555), and 1 μ g/mL recombinant TIMP-1 (Abcam ab82104) treatments are given after microtissues have been allowed to settle for 30 minutes and 1 mL of media is added to every hydrogel.

3.3.4 3D Fixed Microtissue Clearing

Uncleared microtissues are washed in 1x PBS for 1 hour. If stained for immunofluorescence imaging, immunofluorescence labeling protocols are performed before clearing. Uncleared microtissue controls are switched into fresh 1x PBS, while ScaleA2 cleared samples are incubated in the components listed in Table S3.1. CUBIC-2 cleared microtissues are incubated in 50% (w/w) sucrose; 25% (w/w) urea; 10% (w/w) triethanolamine. For incubations longer than 3 hours, samples are kept at 4°C prior to imaging. Before imaging, samples are switched into fresh clearing media.

3.3.5 THP-1 Differentiation, Priming, Nanomaterial Uptake, and Cathepsin-B Staining

To evaluate macrophage phagocytosis, 24-well glass-bottom plates (Eppendorf 0030741021) were coated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO) according to manufacturer's protocol. 200,000 macrophages were plated in the poly-L-lysine-coated wells in media containing 1 ng/mL LPS and 5 μ g/mL nanomaterials in 200 μ L per well. Cells were incubated at 37°C and 5% CO₂ for 4 hours to complete priming. After priming, the medium was removed and replaced with fresh

microtissue cell culture medium containing 5 $\mu\text{g}/\text{mL}$ of each nanomaterial. After 24 hours, THP-1 cell populations were stained according to Magic Red cathepsin-B kit (Bio-Rad ICT937) protocols. Samples were imaged on the Opera Phenix spinning disk confocal fluorescence microscope.

3.3.6 Masson’s Trichrome Histology

For histological evaluation, 5-day 3D lung microtissues were fixed in 10% neutral buffered formalin (Fisher Scientific) in agarose hydrogels for a minimum of 24 hours prior to further processing. Microtissues were processed for embedding in paraffin and processed by the LMM Molecular Pathology Core for 6 μm sectioning and Masson’s trichrome staining for histological examination using brightfield light microscopy.

3.3.7 Fluorescence Imaging

To evaluate the presence of α -SMA, cytokeratin, E-cadherin, vimentin protein endpoints, cells were prepared, cultured, and exposed to exogenous TGF- β 1 or nanomaterials according to protocols stated above. Cells were fixed in 10% neutral buffered formalin and incubated at 4°C overnight before processing. Fixed samples were permeabilized in 0.25% Triton X-100 and blocked with blocking buffer for 3 hours in 5% BSA, 5% goat serum (Sigma G9023), 0.1% Triton, and 0.3 M glycine in 1x PBS. Blocked samples were stained overnight at 4°C with α -SMA (Cell Signaling 19245, 1:250), Cytokeratin Pan Antibody (Thermo Fisher MA5-13203, 1:100), E-cadherin (Cell Signaling 14472S), Vimentin (Cell Signaling, 5741S) antibodies. Primary antibodies were fluorescently labeled with goat anti-rabbit (AF488, Cell Signaling 4412) and goat anti-mouse (AF647, Cell Signaling 4410) at a final dilution of 1:1000. Control wells only received 1:1000 secondary antibody solutions. Samples were counterstained with 1:1000 Hoechst 33342 (Thermo Fisher 62249) and 1:100 rhodamine-phalloidin (Thermo Fisher R415) and imaged on an Opera Phenix spinning disk confocal mi-

croscope (Perkin Elmer). Microtissue α -SMA labels were imaged using a 647 nm excitation laser with a 100 ms exposure time and 20% laser power for fibroblast and macrophage coculture microtissues and with a 80 ms exposure time and 100% laser power for epithelial cell and fibroblast coculture microtissues. 5 μ m microtissue Z-stacks were evaluated using either 20x (still in agarose hydrogels) or 40x (dumped microtissues) water objectives optimized by plate type and microtissue distance from the bottom of the plate.

3.3.8 Confocal Fluorescence Imaging of 3D Microtissue Assembly With Collagen Type 1

Cells were stained with CellTracker Deep Red, CellTracker Green CMFDA, or Hoechst 33342 (Molecular Probes/Thermo Fisher Scientific) using the manufacturer’s protocol prior to assembly into microtissues to evaluate 3D fibroblast microtissue assembly. The labeled cells were imaged in live microtissues using confocal fluorescence microscopy. For volumetric and time-lapse formation analysis, live microtissues were imaged on an Opera Phenix spinning disk confocal microscope. Z-stacks of intact microtissues were obtained using confocal fluorescence microscopy every 5 hours over 70 hours. Imaging conditions were as follows, 100 ms exposure time, 20% laser power, and 3 μ m plane distance across 160 μ m for all stains using 647 nm, 488 nm, and 405 nm excitation lasers.

3.3.9 Microtissue Viability

Hydrogels containing microtissues are washed in 1x PBS or treated with 70% ethanol for 1h. After 1h, microtissues were stained according to LIVE/DEADTM Cell Imaging Kit (Thermo Fisher R37601) protocols with 1:1000 Hoechst 33342 (Thermo Fisher 62249) for 1 hour prior to imaging on the Opera Phenix high-content fluorescent

microscope. Calcein AM stain, indicating live cells, was imaged using a 488 nm excitation laser with a 10 ms exposure time, and 5% laser power. Hoechst stain, indicating cell nuclei, was imaged using a 405 nm excitation laser with a 20 ms exposure time, and 5% laser power. Ethidium Homodimer-1 stain, indicating dying cell nuclei, was imaged using a 568 nm excitation laser with a 50 ms exposure time, and 20% laser power.

3.3.10 Protein isolation and Luminex bead assays

3D microtissue samples were collected by centrifugation at 1000 rpm for 5 minutes at 4°C and rinsed twice with PBS. Pelleted samples were lysed on ice in PBS containing 0.1% Triton X-100, 1 mM EDTA, and cOmpleteTM Protease Inhibitor Cocktail (Millipore Sigma, USA) followed by centrifugation to remove cell debris. The total protein concentration of each sample (mg/mL) was determined using a Bio-Rad DC Protein Assay kit (Bio-Rad, USA). For Luminex (USA) bead assays, 3-plex (TGF- β 1, TGF- β 2, and TGF- β 3) and 4-plex (TNF- α , IL-1 α , IL-1 β , IL-1RA) magnetic bead kits, including reagents, detection antibodies, and standards, were assembled by mixing pre-coupled magnetic beads selected from the MILLIPLEX MAP TGF β Magnetic Bead 3 Plex Kit (Millipore) and Human Cytokine Screening (Cat 12007283) multiplex panels. All protein samples were assayed in duplicate using kit protocols supplied by the manufacturer. Data collection was performed on a Bio-Rad BioPlex 200 plate reader employing BioPlex Manager software. Final analyte levels (pg/mL) were corrected for total protein in each sample and reported as picogram of analyte per milligram of total protein.

3.3.11 Statistical Analyses

All data are reported as mean and standard deviation (SD) unless otherwise stated. Volumetric data were analyzed using one-way ANOVA with a Dunnett's multiple comparisons test using GraphPad Prism 6 software.

3.4 Results and Discussion

3.4.1 Characterizing Models of Cell-Cell Interactions in the Context of Pulmonary Fibrosis

Cell culture on infinitely stiff substrates results in strong cell-substrate adhesion that drives the cytoskeletal organization and masks changes induced by particle exposure. Alternatively, cell culture on soft substrates reveals subtle changes to cytoskeletal architecture that can be quantified to identify population-level changes driven by particle exposure that is not quantified by phenotypic biomarkers alone. While different 3D models of fibrosis have been developed using multiple 3D culture platforms, most models include cell culture substrates, are not cost-effective, reproducible or readily available, and are not evaluated for efficacy in fibrous and graphene particle nanomaterial screening. To develop models that meet the aforementioned practical needs and with the ability to measure cell-cell and cell-matrix interactions in scaffold-free conditions, several non-adherent microtissue models were developed and characterized (Figure 3.1). Three types of microtissues were developed in an attempt to characterize human lung cell response to particles, including 1) an IMR-90 fibroblast microtissue, 2) an IMR-90 fibroblast microtissue with collagen ECM protein, 3) a model of the fibroblast-macrophage axis using hTERT immortalized primary fibroblasts and THP-1 macrophages, and 4) a model of the fibroblast-epithelial cell axis using IMR-90 fibroblasts and BEAS-2B epithelial cells. Figure 3.1 illustrates the

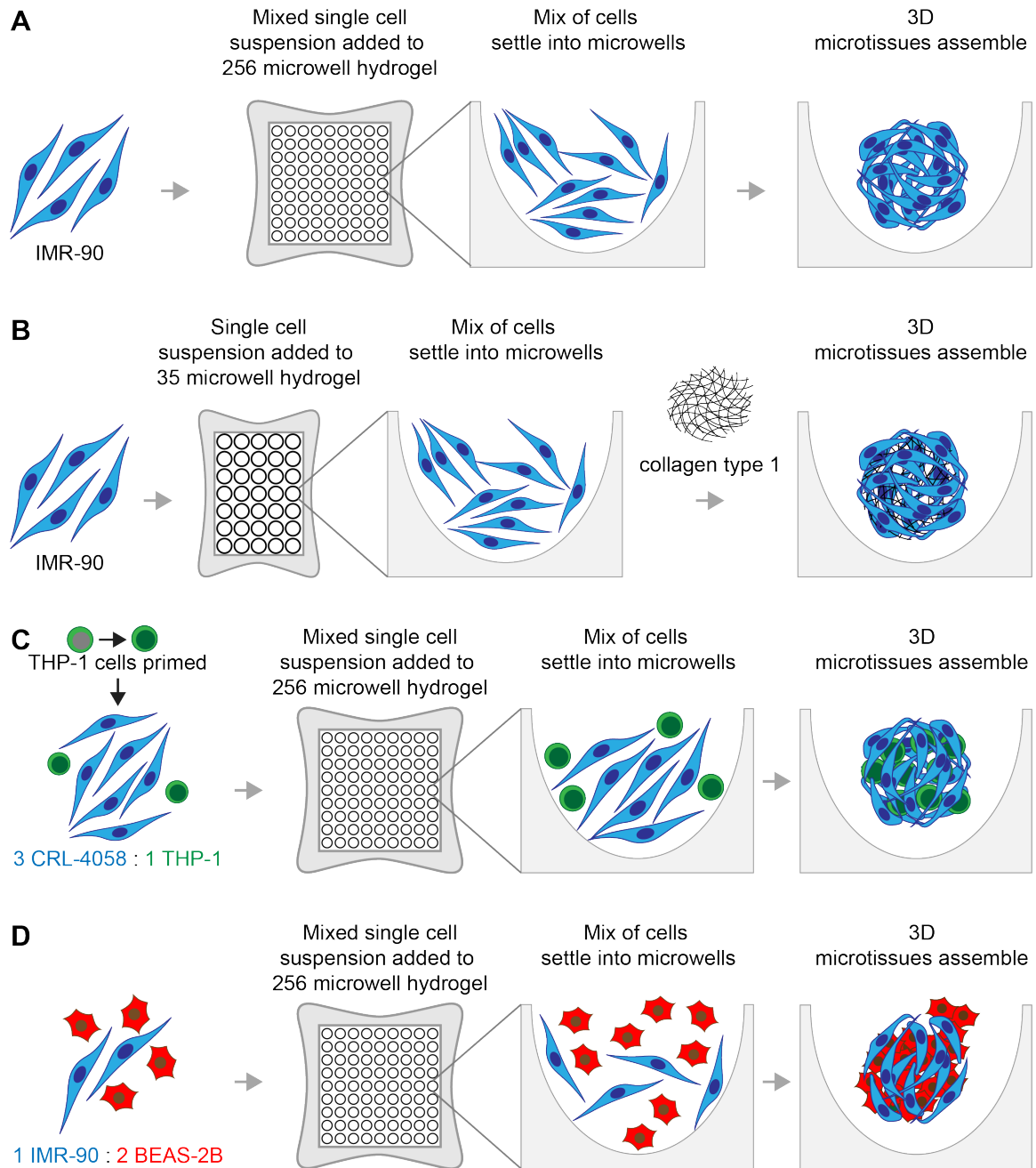


Figure 3.1: 3D Non-adherent Microtissue Plating Paradigms.

(A) IMR-90 fibroblast microtissues, (B) IMR-90 fibroblast microtissues with a collagen scaffold, (C) and hTERT-immortalized primary human lung fibroblast and THP-1 macrophage coculture microtissue, (D) IMR-90 fibroblast and BEAS-2B epithelial cell coculture microtissues.

non-adherent microwells cell culture conditions in which single-cell suspensions settle over time and self-assemble into microtissues.

Optimizing Microtissue Clearing Techniques

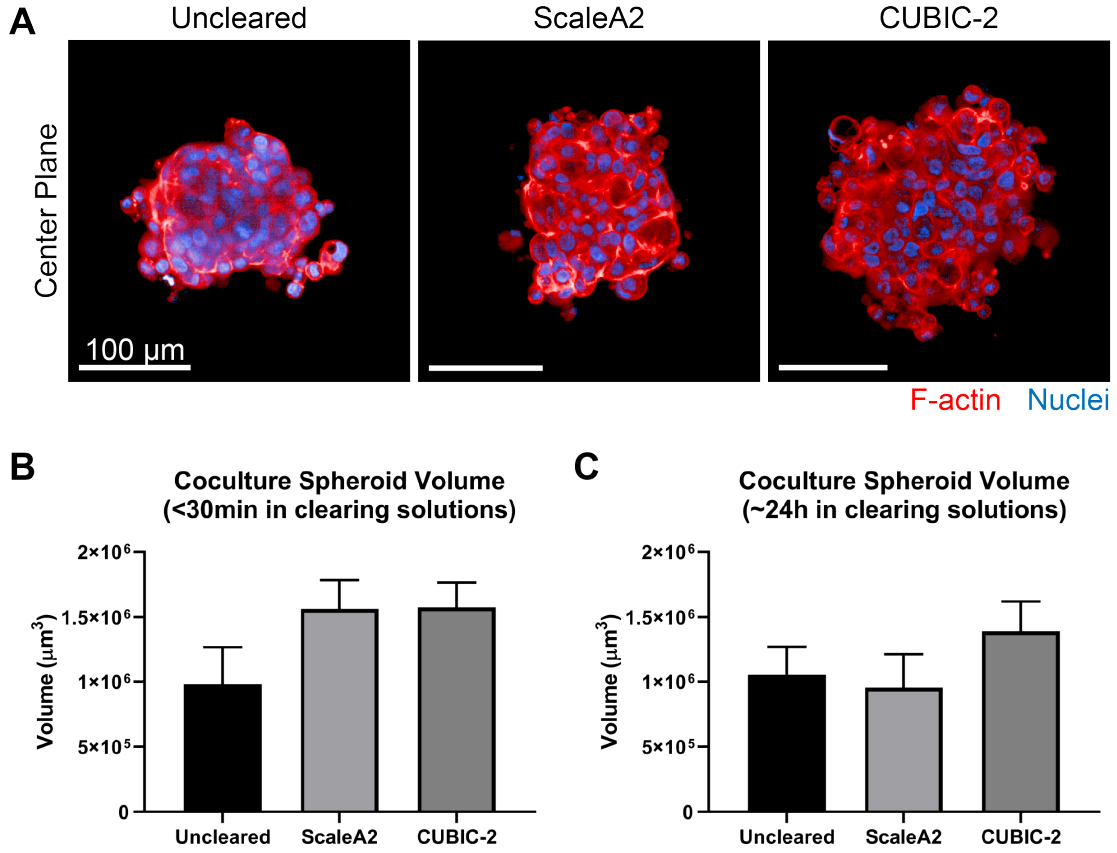


Figure 3.2: Clearing Optimization.

Several clearing solutions were evaluated for their efficacy in improving whole-microtissue fluorescence imaging while minimizing shrinkage or swelling. (A) Microtissues labeled with nuclear and filamentous actin stains appear morphologically similar after a 24 hour incubation in clearing solutions. Nuclear and actin signals are blurred at the microtissue center in uncleared microtissues. (B) Initial equilibration in clearing solutions causes microtissue swelling. (C) Microtissues cleared in ScaleA2 solution for 24 hours return to uncleared microtissue volume but not Cubic-2 cleared samples. Experiments were performed across three, 256-microwell hydrogels and at least 50 microtissues per hydrogel were quantified.

To evaluate changes in whole microtissues through immunofluorescence imaging, clearing solutions were tested to maximize whole microtissue imaging capabilities and minimize destructive shrinkage, swelling, and maximum staining and antibody label preservation. Initial experiments tested the efficacy of two clearing solutions,

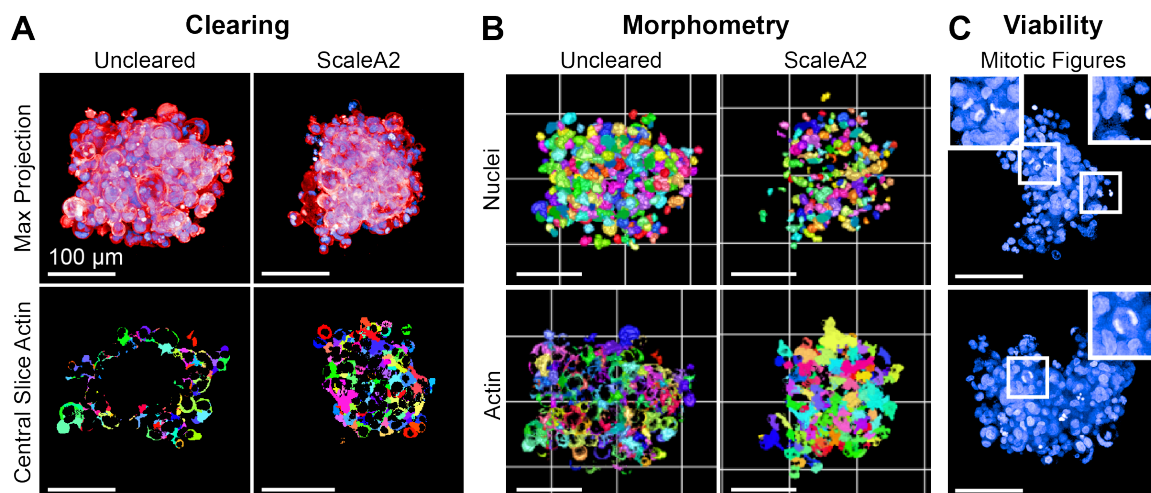


Figure 3.3: ScaleA2 Clearing.

(A) ScaleA2 clearing solution improves visualization of central filamentous actin organization compared to uncleared microtissues. (B) ScaleA2 clearing increases resolution of nuclei and actin in 3D microtissue renderings, resulting in decreased nuclear size and increased actin within the microtissue body. (C) ScaleA2 clearing allows for visualization of mitotic figures throughout microtissues.

ScaleA2 and CUBIC-2. Microtissues were incubated in 1x PBS, ScaleA2, or CUBIC-2 clearing solutions, imaged, and visually evaluated using a central slice from each Z-stack (Figure 3.2A). Initial incubation in each clearing solution resulted in microtissue swelling (Figure 3.2B). After 24 hours, CUBIC-2 microtissues remained swollen (Figure 3.2C). ScaleA2 clearing allowed for imaging of whole microtissues (Figure 3.3A) with the required resolution to evaluate changes in nuclear size and clustering (Figure 3.3B), single cell cytoskeletal organization (Figure 3.3B), and visualize mitotic figures (Figure 3.3C).

3.4.2 IMR-90 Fibroblast Microtissues

IMR-90 microtissues are not viable after 72 hours.

IMR-90 fibroblasts underwent fibroblast to myofibroblast differentiation in 2D cell culture on stiff and soft substrates when exposed to TGF- β 1. However, fibroblasts in

homeostatic lung microenvironments maintain the pulmonary architecture without experiencing activation, proliferation, and differentiation responses that characterize wound healing activity. Fibroblastic foci are features of fibrotic microenvironments comprised of clusters of fibroblasts and myofibroblasts that ultimately destroy the pulmonary architecture.

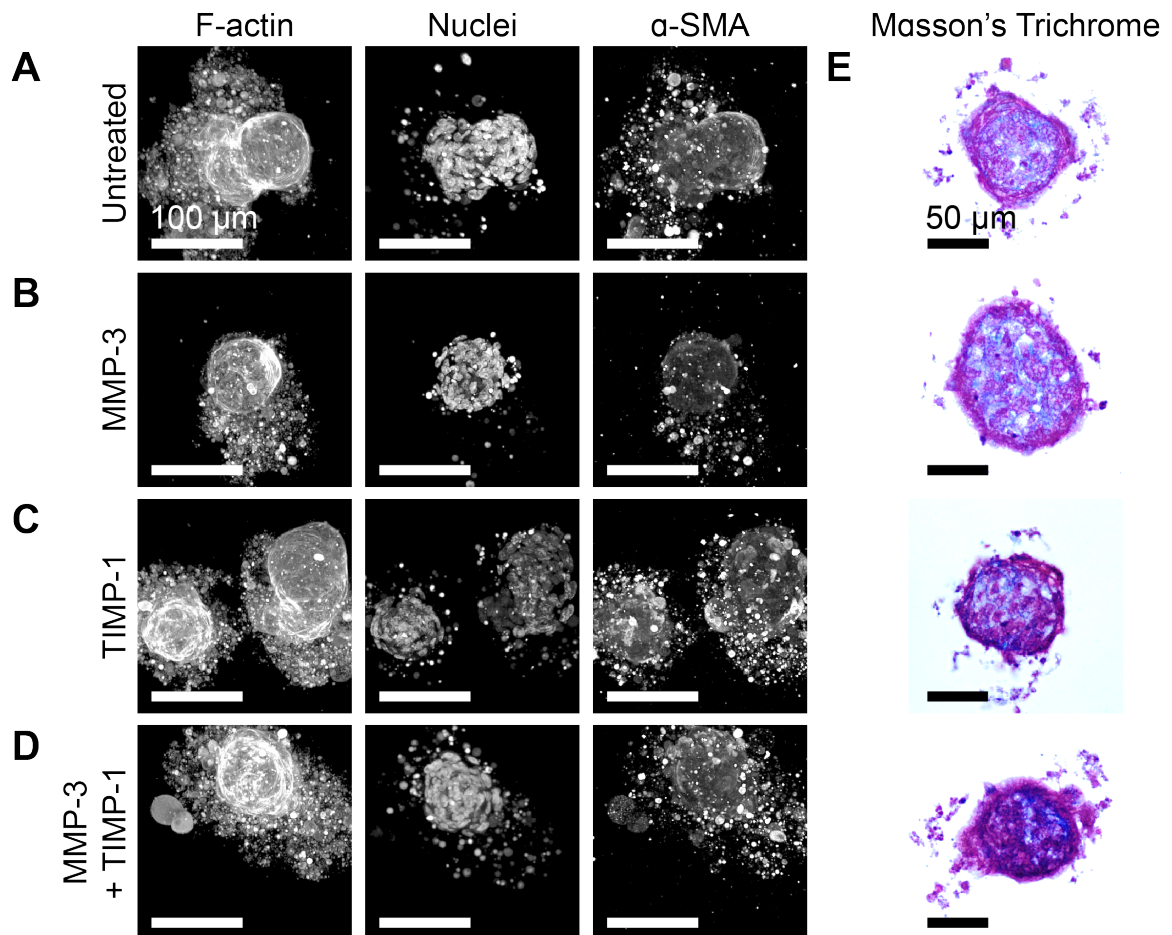


Figure 3.4: IMR-90 Fibroblast Microtissue Morphological and Phenotypic Characterization

(A) Untreated IMR-90 fibroblast microtissues seeded without ECM do not remain viable, indicated by excessive cell debris. (C) Microtissues were treated with recombinant TIMP-1 to decrease the degradation of ECM by the MMP family. TIMP-1 treatment was compared to (B) recombinant MMP-3 and (D) combination MMP-3 and TIMP-1 treatment to confirm that morphological changes were specific to MMP family modulation. No changes to morphology or fibroblast phenotype were observed. (E) No changes to ECM deposition (blue) is observed in representative sections of microtissues stained by Masson's trichrome staining.

In an attempt to model a fibroblastic focus, IMR-90, human lung fibroblasts were cultured in non-adherent hydrogels, forming loosely-associated clusters of fibroblasts (Figure 3.1A). The fibroblast-derived microtissue was intended to evaluate particle-driven differentiation in regions of particle aggregation driven solely by cell-cell and cell-particle interactions in the absence of an adherent substrate. However, fibroblast-only microtissues were not viable, disintegrating into cell debris with a small, viable cell cluster after 72 hours. The remaining microtissue body was comprised of myofibroblasts (Figure 3.4). As a result, we hypothesized that decreasing nascent ECM remodeling would result in increased ECM deposition within the microtissue and could promote viability. To modulate the degradation of the ECM, microtissues were cultured with recombinant Tissue Inhibitor Of Metalloproteinases-1 (TIMP-1) for 72 hours (Figure 3.4C). Recombinant MMP-3 (Figure 3.4B), MMP family enzyme that can degrade fibronectin, laminin, collagens III, IV, IX, and X, treatment and combinatorial treatment with MMP-3 and TIMP-1 (Figure 3.4D) controls were employed and compared to untreated fibroblast microtissues (Figure 3.4A) to confirm regulation of the MMP family and interpret any changes to microtissue viability or ECM deposition. No changes to microtissue viability or ECM deposition were observed.

IMR-90 microtissues with collagen are viable and stable after 72 hours.

Rat-tail collagen type 1 was mixed with the fibroblast cell suspension to provide IMR-90 fibroblasts with a scaffold material that could integrate into microtissues during formation (Figure 3.1B). When two cell types are mixed, cell sorting can be readily observed and indicate a baseline timepoint at which microtissues can be treated to observe phenotypic deviation from baseline. However, with only one cell type, microtissue formation and stability after 3 hours were difficult to observe by eye. To evaluate microtissue stability, three populations of IMR-90 fibroblasts were stained with either nuclear or CellTracker stains and mixed at a 1:1:1 ratio. IMR-

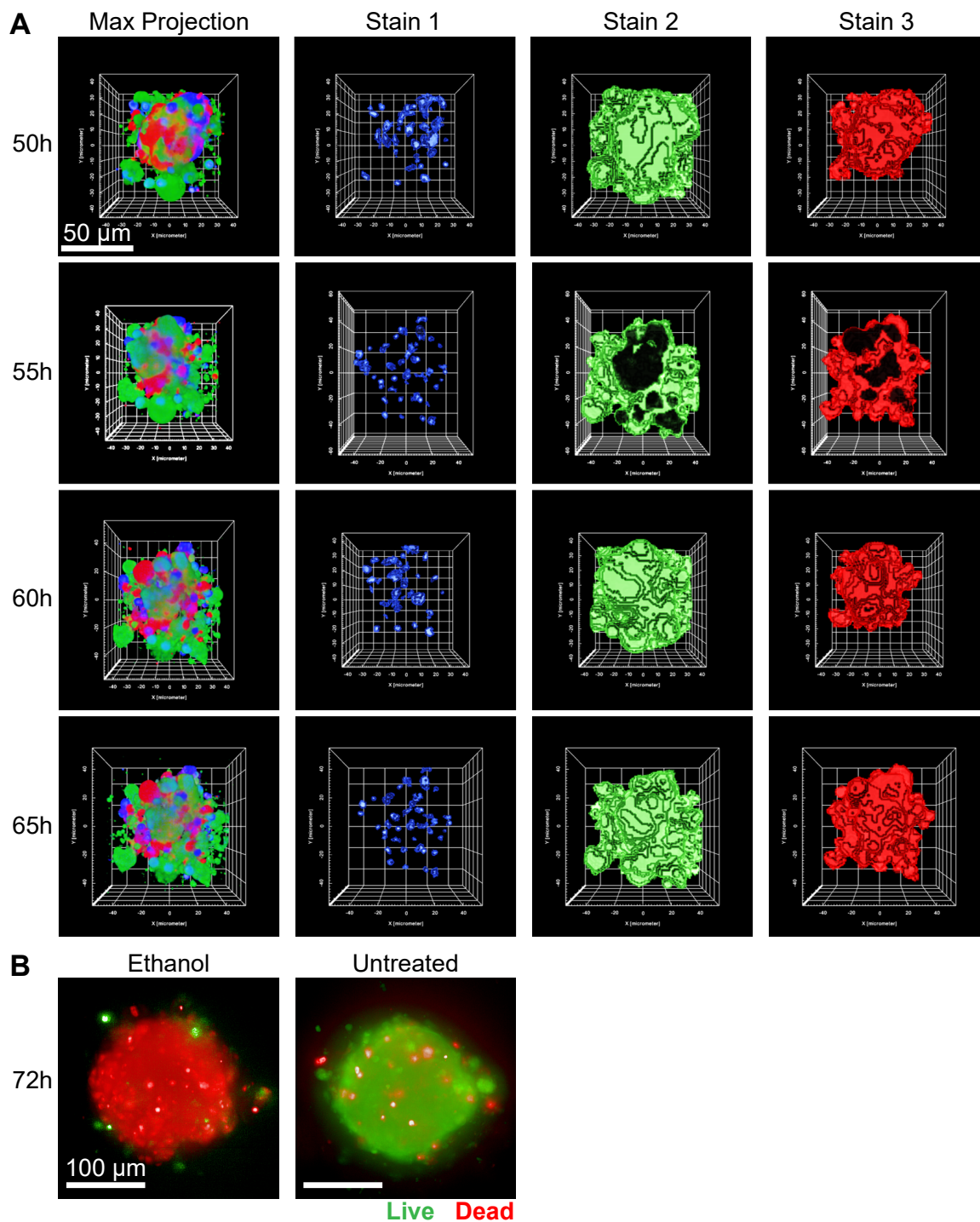


Figure 3.5: IMR-90 Fibroblast Microtissues Remain Viable After 72 Hours. (A) Three populations of fibroblasts were separately stained and mixed in equal parts during seeding and observed for organization within the untreated microtissue over 72 hours. Stable microtissue formation occurs between 55- and 60 hours. (B) IMR-90 fibroblast microtissues with collagen are viable after 72 hours.

90 microtissue formation around collagen stabilized, on average, between 55- and 60 hours after seeding (Figure 3.5A) and informs a starting timepoint for microtissue evaluation in later experiments. Fibroblast microtissues with collagen were viable after 72 hours and had less cell debris (Figure 3.5B). After attempts to collect RNA and protein from this microtissue model yielded low concentrations, further efforts to characterize this model at a molecular level were not pursued in favor of other models.

3.4.3 Primary Fibroblast and Macrophage Microtissues

Differentiated and primed THP-1 macrophages phagocytose control nanomaterials and exhibit M1 macrophage phenotypes.

Resident and recruited macrophages phagocytose and attempt to clear deposited nanomaterials and are the primary producers of TGF- β 1 during fibrogenesis. As a result, clusters of fibroblasts often associate with macrophages in the fibrotic microenvironment. However, Hinz et al. found that physical interactions between fibroblasts and macrophages through cadherin-11 also directly regulate the secretion of TGF- β 1 by both cell types [56]. However, the platform on which the research was performed required a fluid flow system. To make this groundbreaking research more accessible, we aimed to translate cell culture in the microfluidic system into the non-adherent microtissue environment using hTERT-immortalized CRL-4058 primary lung fibroblasts and THP-1-derived macrophages (Figure 3.1C).

The incorporation of macrophages into the microtissue model introduces a restricted form of clearance. While macrophages could not be removed from the hydrogel to perform functional clearance, we confirmed that THP-1 macrophages were able to fully or partially phagocytose all particles of the chosen particle panel (Figure 3.6) and exhibit rounded and elongated, adherent morphologies (Figure 3.7A). Untreated macrophages and macrophages exposed to carbon black and asbestos have

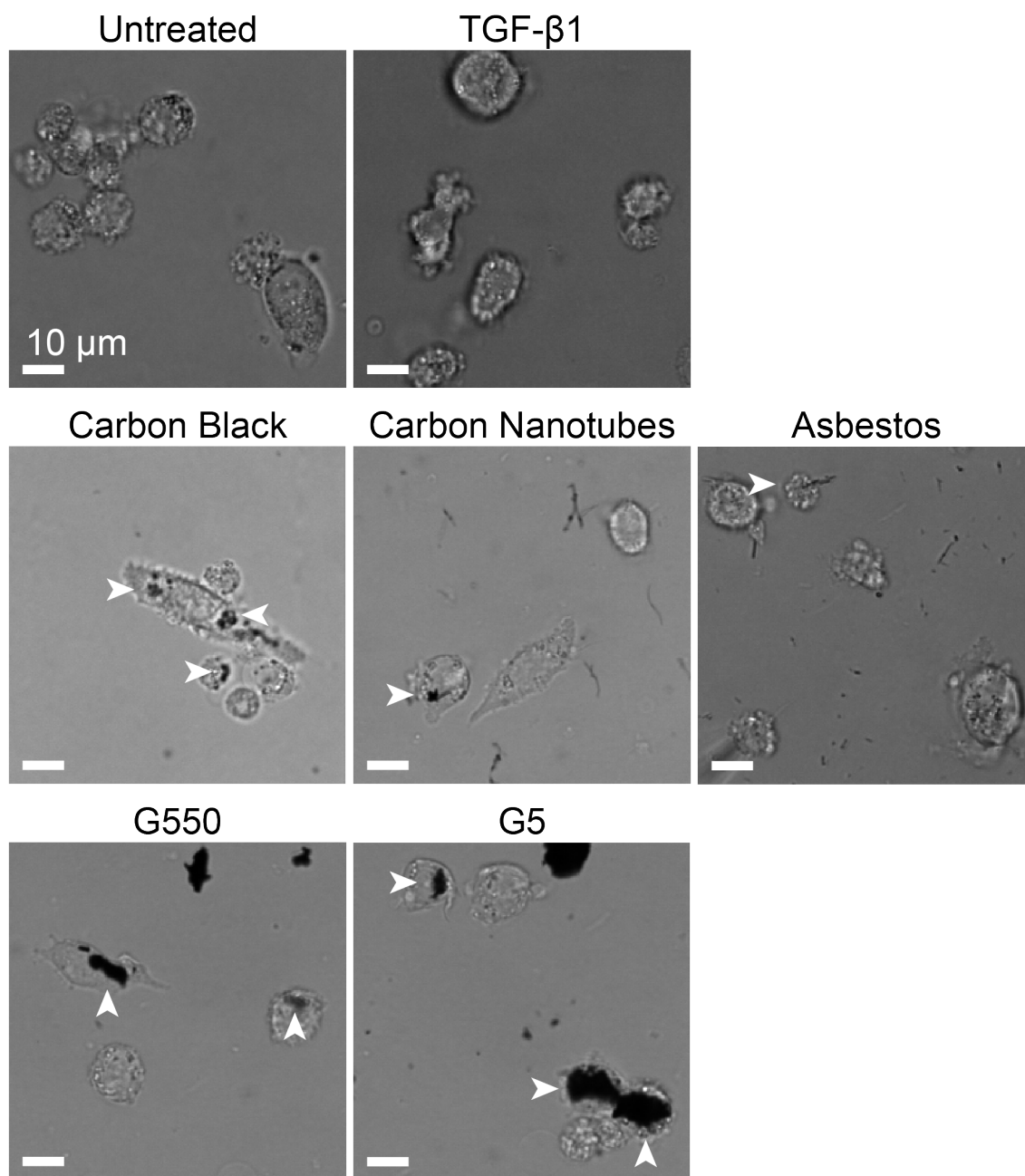


Figure 3.6: THP-1 Macrophages Phagocytose Nanomaterials. Differentiated and primed THP-1 macrophages exhibit rounded and adherent, elongated morphologies. Exposure to all particles results in particle uptake and sequestration within macrophages (white arrows).

intact lysosomes filled with cathepsin-B. Carbon nanotube exposure caused lysosomal damage, leading to cathepsin-B leakage in most cells (Figure 3.7B). Finally, untreated macrophages were characterized as M1 macrophages by cytokine profiles that were

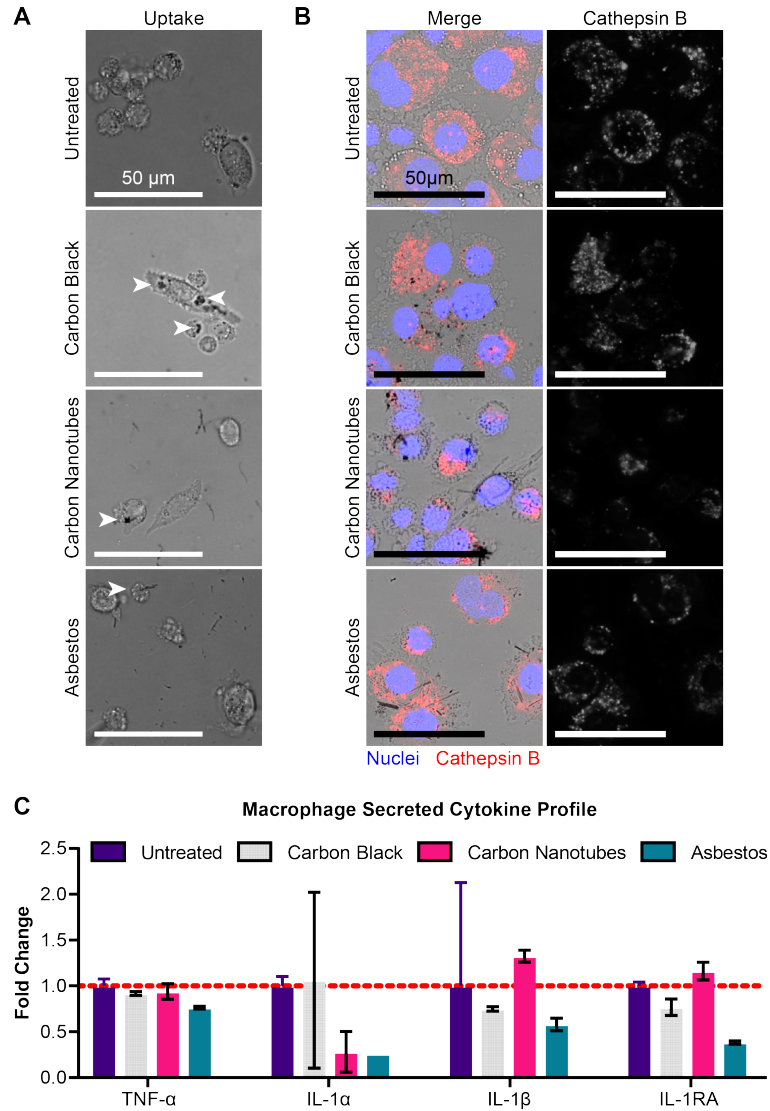


Figure 3.7: THP-1 Macrophage Characterization.

(A) Differentiated and primed THP-1 macrophages phagocytose control particles (white arrows) and exhibit rounded and elongated, adherent morphologies after 24 hours. (B) Cathepsin-B staining identifies intact lysosomal compartments. After 24-hour particle exposure, carbon nanotube exposure results in lysosomal damage characterized by loss of punctate lysosomal compartments containing cathepsin-B that is not observed after carbon black and asbestos exposure. (C) THP-1 conditioned media was collected after a 24-hour exposure to control particles. M1 cytokine expression profiles indicate marginal increases in cytokine secretion in response to carbon nanotube exposure and decreased cytokine production after asbestos exposure. Statistics were performed using a two-way ANOVA with a Dunnett's post-test. The red dotted line indicates the untreated average for reference.

similar to carbon black and carbon nanotube exposures but experienced decreasing trends in cytokine production after asbestos exposure (Figure 3.7C).

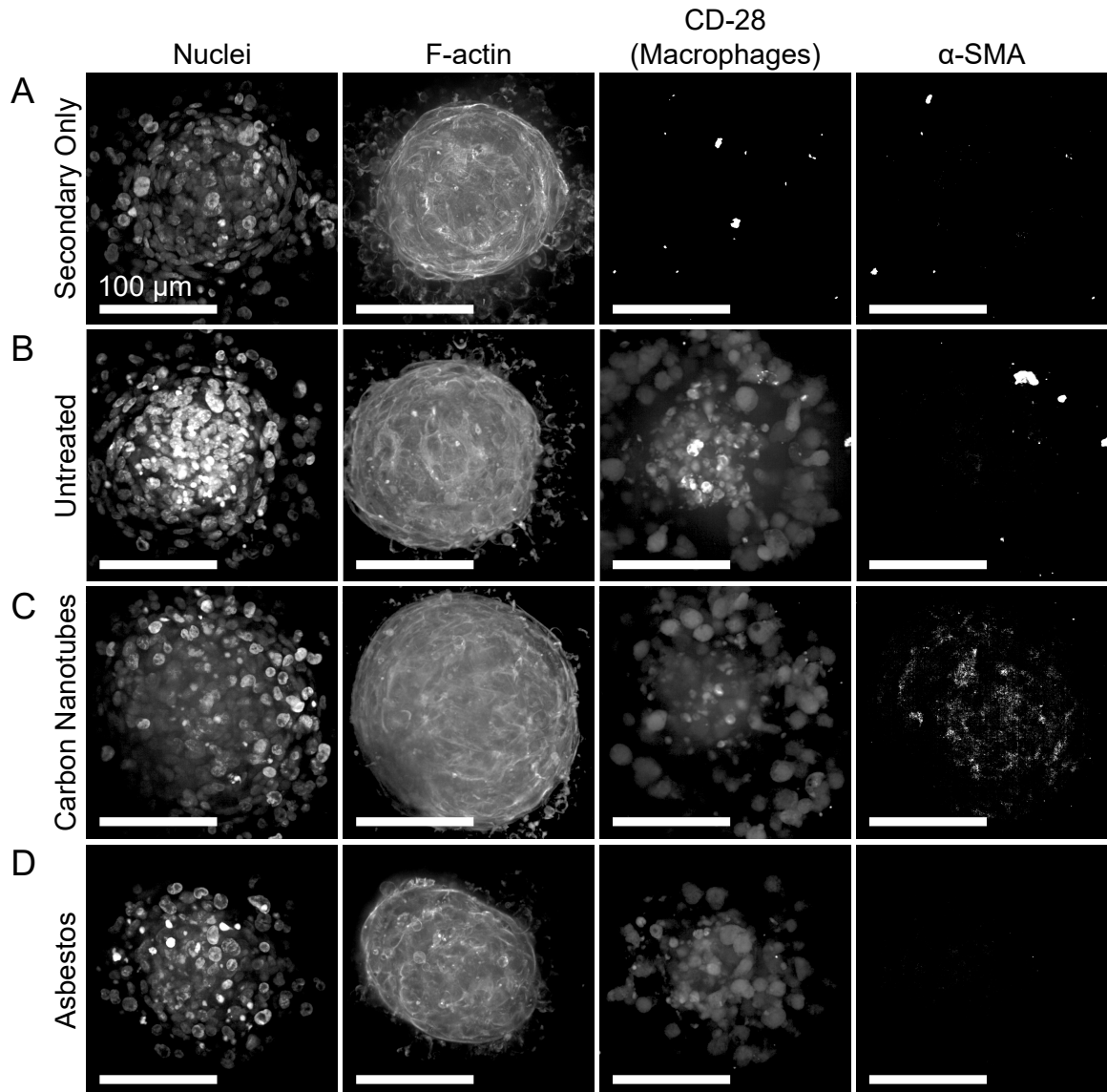


Figure 3.8: Primary Fibroblast and Macrophage Microtissue Organization
Microtissues are seeded, fixed at 4-days, and stained using nuclear and actin stains and labeled with CD-28 macrophage and α -SMA antibodies. (B) Untreated microtissues are comprised of a dense central body containing a central cluster and halo of macrophages with fibroblasts forming a layer around the center macrophage cluster. The fibroblasts remain undifferentiated in the microtissue body. (C) Carbon nanotube and (D) asbestos incorporation into the microtissue body does not alter cell sorting, but carbon nanotube exposure may increase α -SMA expression. (A) Representative images of secondary-only antibody controls.

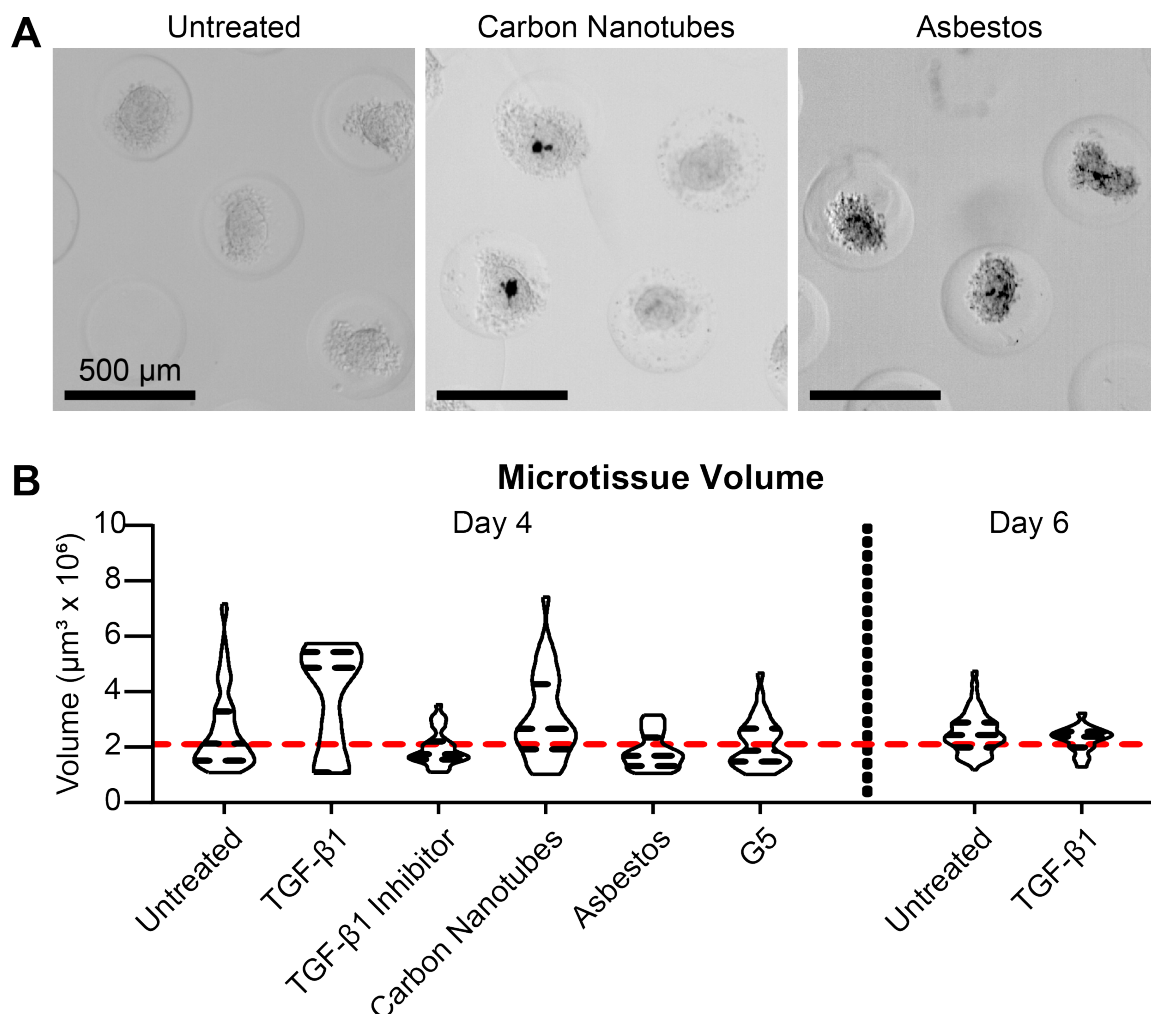


Figure 3.9: Primary Fibroblast and Macrophage Microtissue Particle Incorporation and Morphology

(A) Microtissues formed compact cell centers surrounded by loosely-associated macrophages. Fibrous particle exposure results in aggregation and incorporation of particles into the microtissue body. (B) Preliminary microtissue volume quantification shows no significant differences in microtissue volume between conditions with increased size consistency over time. The red dotted line indicates the untreated median for reference.

Carbon nanotube exposure increases expression of α -SMA in fibroblast and macrophage microtissues.

Primary fibroblast and macrophage single-cell suspensions form compact microtissues characterized by a central core of macrophages and a dense shell of primary fibrob-

lasts, accompanied by a loosely-associated halo of macrophages (Figure 3.8AB). After exposure to carbon nanotubes (Figure 3.8C) and asbestos (Figure 3.8D), microtissue sorting remains consistent with untreated microtissues, but increased expression of α -SMA occurs in the fibroblast outer shell (Figure 3.8C). Fibrous nanomaterials are incorporated into microtissues as discrete aggregates (Figure 3.9A). Microtissue morphology, though varied, is statistically similar across all conditions after 4- and 6 days (Figure 3.9B).

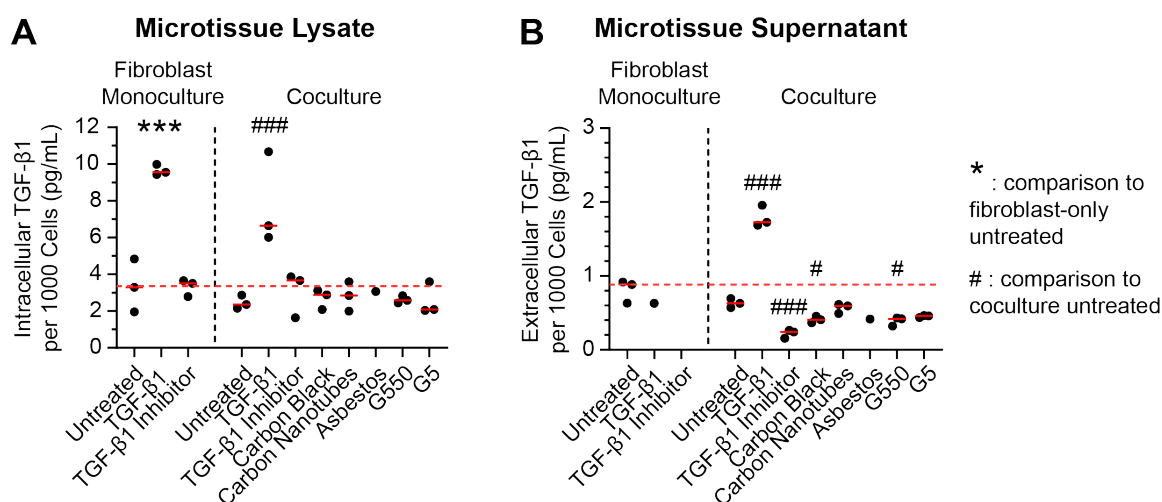


Figure 3.10: Fibroblast and Macrophage Microtissue TGF- β 1 Production and Secretion.

Monoculture and coculture microtissues were compared to their respective untreated microtissues to observe changes in TGF- β 1 abundance driven by nanomaterial exposure. In order to control for the incorporation of recombinant TGF- β 1 into latent complexes or uptake into cells, a TGF- β inhibitor was employed. (A) Coculture microtissue lysates exhibit decreased production of TGF- β 1 compared to primary fibroblast microtissues alone. (B) Microtissue supernatant collections confirm reduced secretion of TGF- β 1, except after carbon nanotube and G5 graphene micro-sheet exposures. Statistics were calculated using a Kruskal-Wallis test with a Dunn's multiple comparisons test (** $p < 0.001$, # $p < 0.05$, ### $p < 0.001$). Red, dotted lines denote monoculture microtissue median values for ease of comparison across conditions. All samples are acidified for the assay to measure free TGF- β 1.

In Hinz et al. 2019, a TGF- β RI inhibitor was used to mediate the positive feedback signaling of secreted TGF- β 1 and resulting fibroblast to myofibroblast differentiation [56]. To remove the physical interaction between fibroblasts and macrophages, pri-

mary fibroblast monoculture microtissues were grown for 4-days and collected for Luminescence TGF- β 1 quantification. Microtissue lysates and supernatants were collected as a baseline measurement of TGF- β 1 production and secretion in 3D non-adherent systems. The addition of recombinant TGF- β 1 significantly increased TGF- β 1 production in primary fibroblast microtissues. Treatment with TGF- β RI inhibitor did not decrease TGF- β 1 production. When cultured together, fibroblast and macrophage microtissues marginally down-regulate production of TGF- β 1 compared to fibroblast microtissues. Furthermore, coculture microtissues do not up-regulate TGF- β 1 production in response to any nanomaterial exposure (Figure 3.10A). Likewise, coculture microtissues did not exhibit increases in secretion of TGF- β 1 after nanomaterial exposure, but TGF- β RI inhibitor did function to decrease secretion compared to untreated coculture microtissues. Decreases in TGF- β 1 associated with carbon black and G550 graphene nano-sheet exposures could be attributed to adsorption of secreted TGF- β 1 or act to decrease TGF- β 1 exposure (Figure 3.10B).

3.4.4 IMR-90 Fibroblast and BEAS-2B Epithelial Cell Microtissues

IMR-90 fibroblasts and BEAS-2B epithelial cells were cultured as 3D scaffold-free microtissues to evaluate the effects of particle exposure on fibroblast and epithelial cell phenotypes in the absence of an artificial substrate (Figure 3.1D).

Baseline epithelial cell phenotypes were quantified using epithelial cell and mesenchymal cell markers in confluent monolayers. BEAS-2B epithelial cells will differentiate into stromal cells if allowed to remain confluent. BEAS-2B cells were plated and fixed at the point of confluence to evaluate epithelial cell phenotypes in conditions of high contact in 2D cell culture. Epithelial cells were exposed to increasing concentrations of recombinant TGF- β 1 and assessed for fibroblast-like phenotypes, epithelial cytokeratin and E-cadherin biomarkers, and mesenchymal marker vimentin.

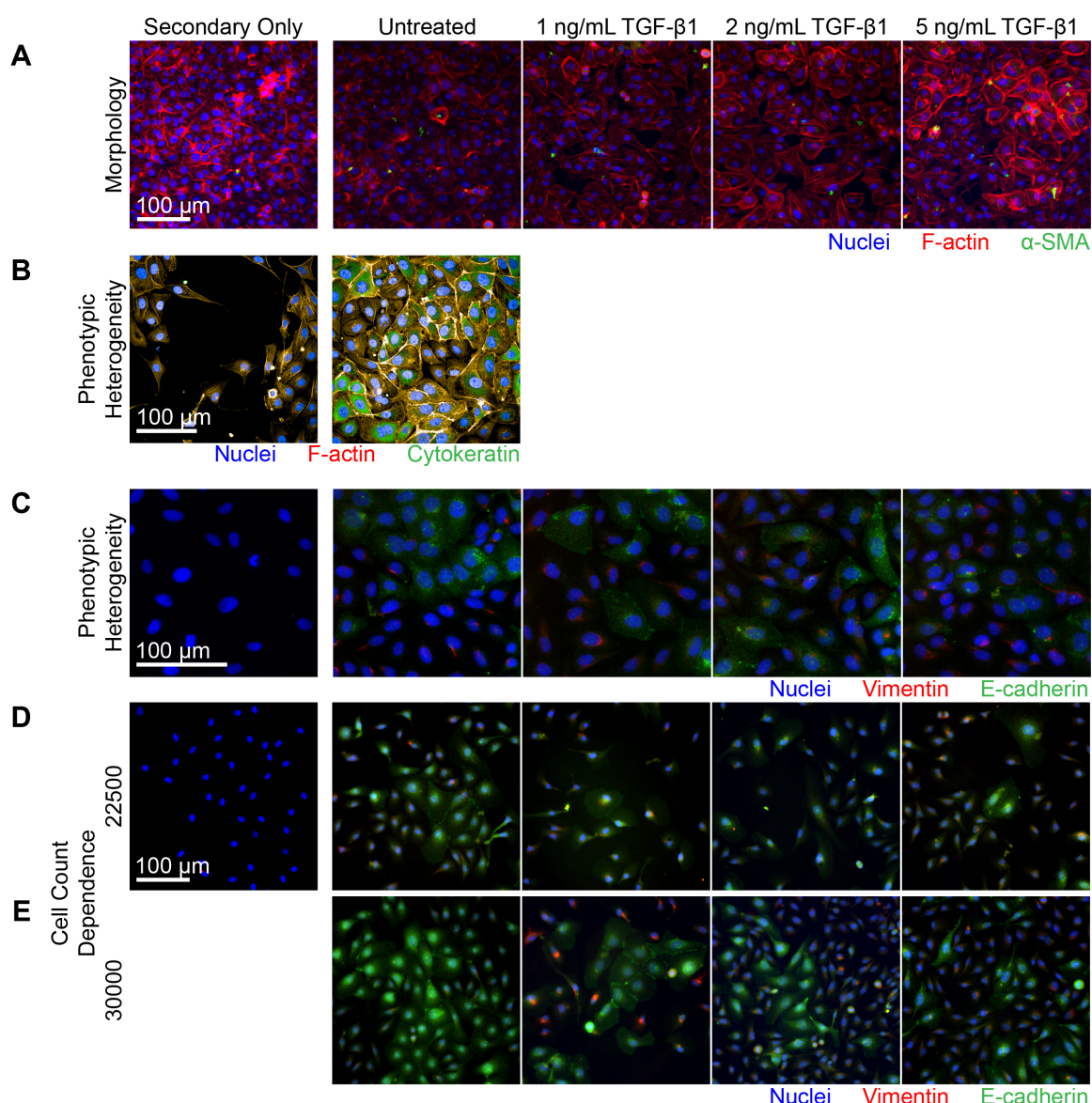


Figure 3.11: Characterizing BEAS-2B Epithelial Cells
 BEAS-2B epithelial cells were cultured for morphological, phenotypic, and cell-cell junction characterization in 2D cell culture conditions meant to mimic maximum contact likely to be experienced in 3D conditions. (A) BEAS-2B cells cultured to confluence over 72 hours. BEAS-2Bs mobilize greater amounts of actin after 5 ng/mL TGFβ1 exposure with some cells exhibiting elongation. 2D confluent cell culture did not induce α-SMA expression. (B) Untreated populations are heterogeneous for cytokeratin and (C) E-cadherin expression. Decreased E-cadherin and increased vimentin expression occurs with increased TGF-β1 concentrations with consistent population heterogeneity across (D) lower and (E) higher cell culture densities.

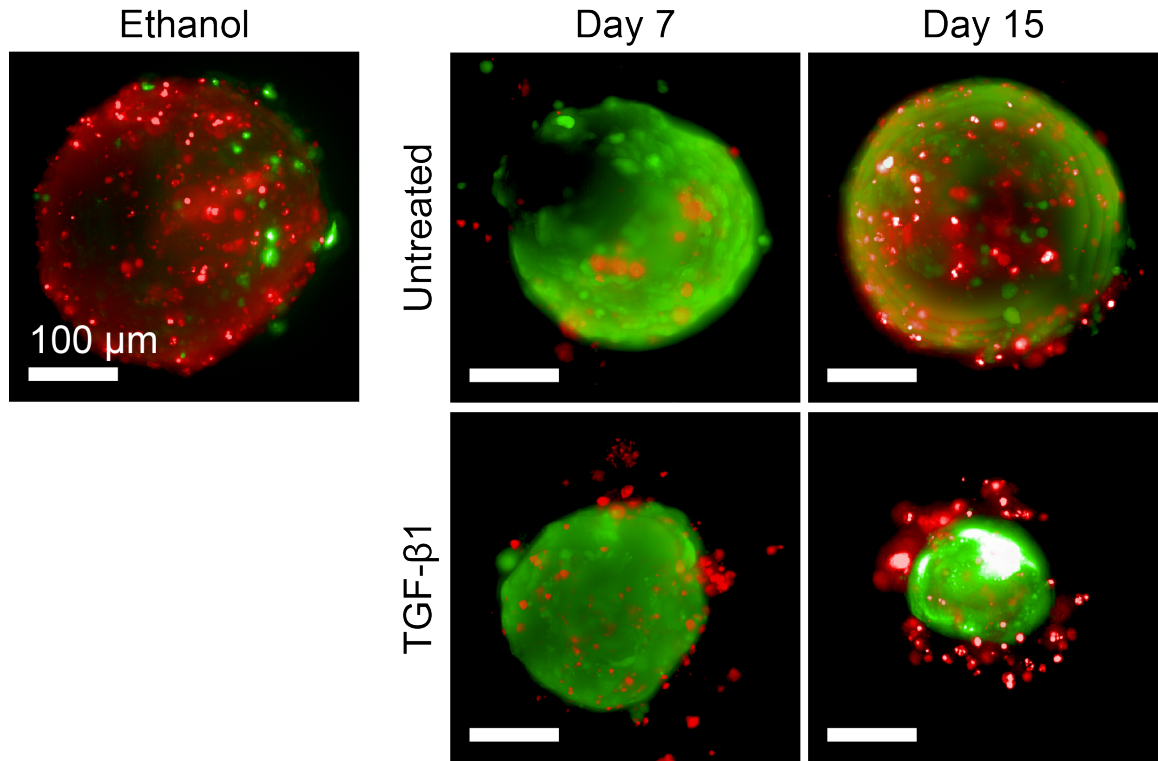


Figure 3.12: Coculture Microtissue Viability Over Time.

Initial viability tests confirm the presence of a compact, live microtissue core (green) in untreated and TGF- β 1-exposed microtissues until 15 days. Untreated microtissues remain viable overall, but TGF- β 1-exposed microtissues have lost the majority of their central mass by 15 days. Dead cells and cell debris (red) collects at the microtissue periphery.

Epithelial cells mobilize increasing amounts of actin after exposure to high concentrations of TGF- β 1 but are negative for myofibroblast marker α -SMA (Figure 3.11A). BEAS-2B epithelial cell populations are heterogeneous for cytokeratin (Figure 3.11B) and E-cadherin expression (Figure 3.11C) with low levels of mesenchymal cell marker vimentin. The number of cells positive for vimentin increase with increasing concentrations of TGF- β 1, but population heterogeneity is consistent across varying confluency (Figure 3.11D, E).

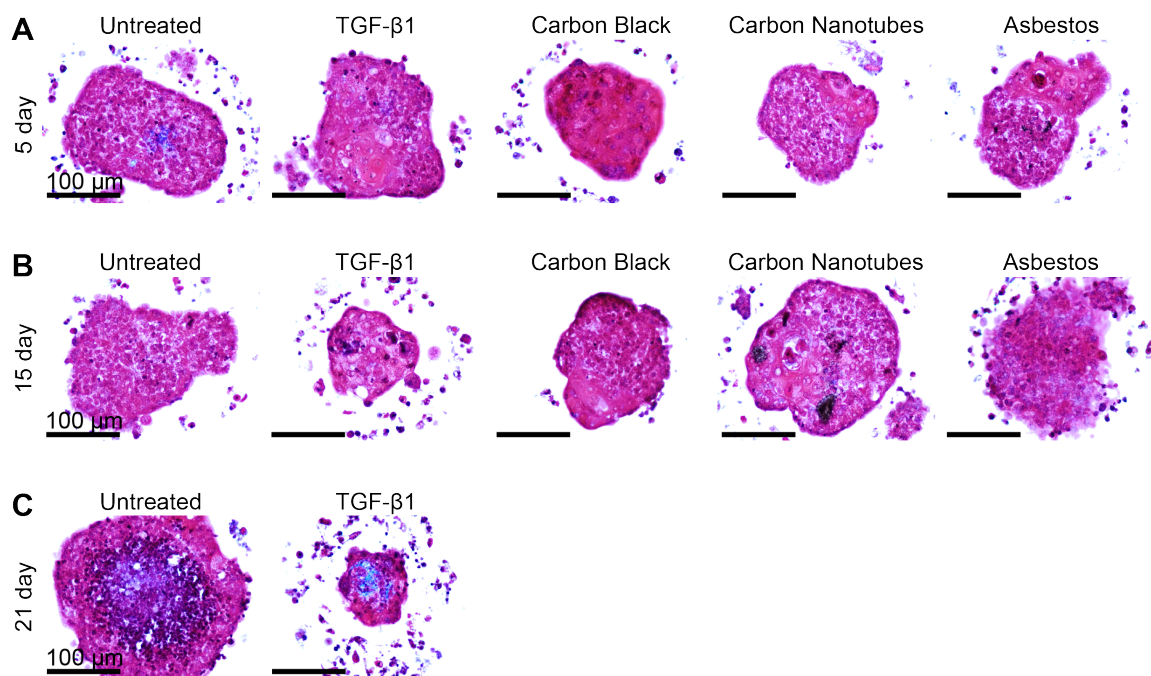


Figure 3.13: Coculture Microtissues Exhibit Minimal ECM Deposition. Microtissues were sectioned and stained with Masson's Trichrome stain to identify nuclei (purple), cytoplasm (pink) and deposited ECM (blue). (A) 5- and (B) 15-day microtissues have minimal deposition of ECM. (B) 15-day microtissues exposed to particles do not exhibit regions of excess ECM, even in microtissues with large carbon nanotube aggregates. (C) 21-day untreated microtissues are large enough to develop hypoxic centers, while TGF- β 1-exposed microtissues are dying.

Use of TGF- β 1 exposure as a positive control limits microtissue use to timepoints below 7 days.

Coculture microtissues have a viable, compact central cluster of cells, but exposure to TGF- β 1 significantly reduced the viable microtissue volume before 15 days (Figure 3.12). To determine if TGF- β 1 exposure was causing increased compaction as a result of increased ECM deposition, microtissues were fixed, embedded, sectioned, and stained for connective tissue by Masson's trichrome staining. Blue staining of ECM identified some dense clusters of ECM within microtissues, with most microtissues exhibiting small amounts of ECM throughout the entire microtissue (Figure 3.13AB). ECM was not observed to be heavily deposited around dark regions in-

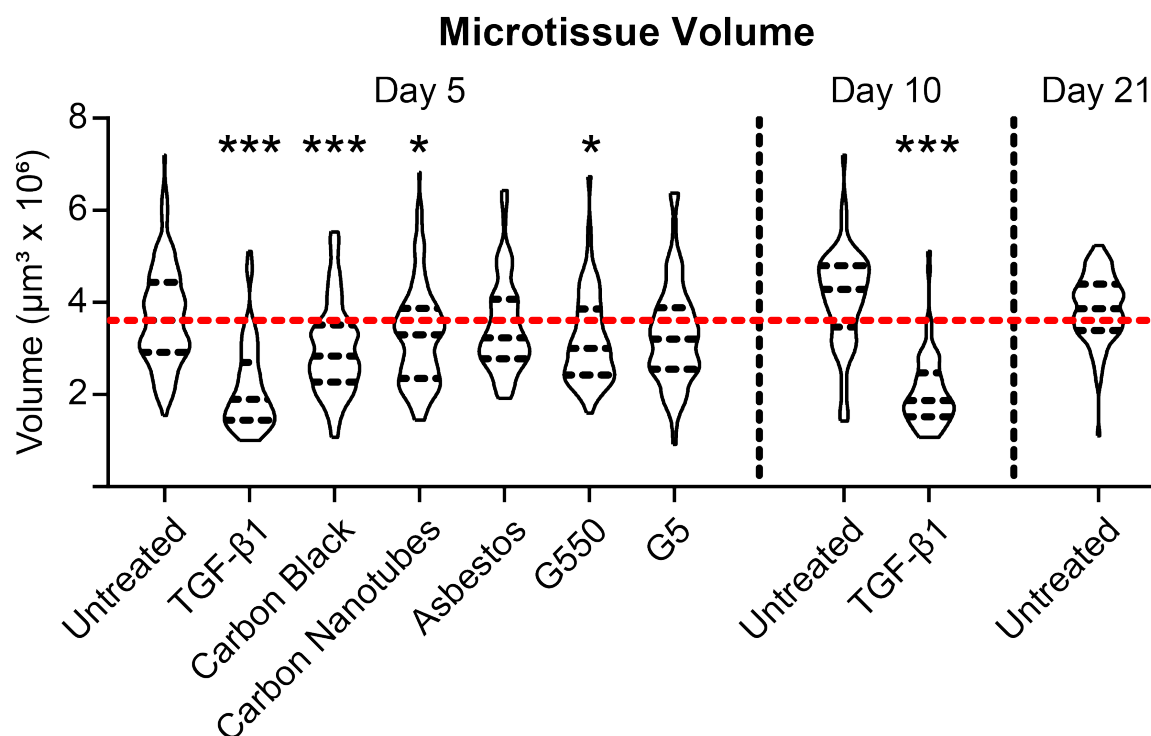


Figure 3.14: Coculture Microtissue Volume Over Time.

Microtissue health for all conditions is most stable before 7 days. Microtissue volume quantified after 5 days shows increased compaction in microtissues exposed to particles. Loss of viability in TGF- β 1-exposed microtissues results in smaller microtissues. Quantification of microtissue stability out to 21 days in untreated and TGF- β 1-exposed microtissues revealed stable untreated microtissues and dwindling TGF- β 1-exposed microtissues. TGF- β 1-exposed microtissues did not survive out to 21 days. Black dotted lines within violin plots, from the top down, indicates the 75th percentile, median, and 25th percentile. The red dotted line indicates the 5-day untreated microtissue median volume for ease of visualization across conditions. Statistics were calculated using a Kruskal-Wallis test with a Dunn's multiple comparisons test (* $p < 0.05$, *** $p < 0.001$). Data is based on microtissue volumes from at least three different hydrogels for each condition.

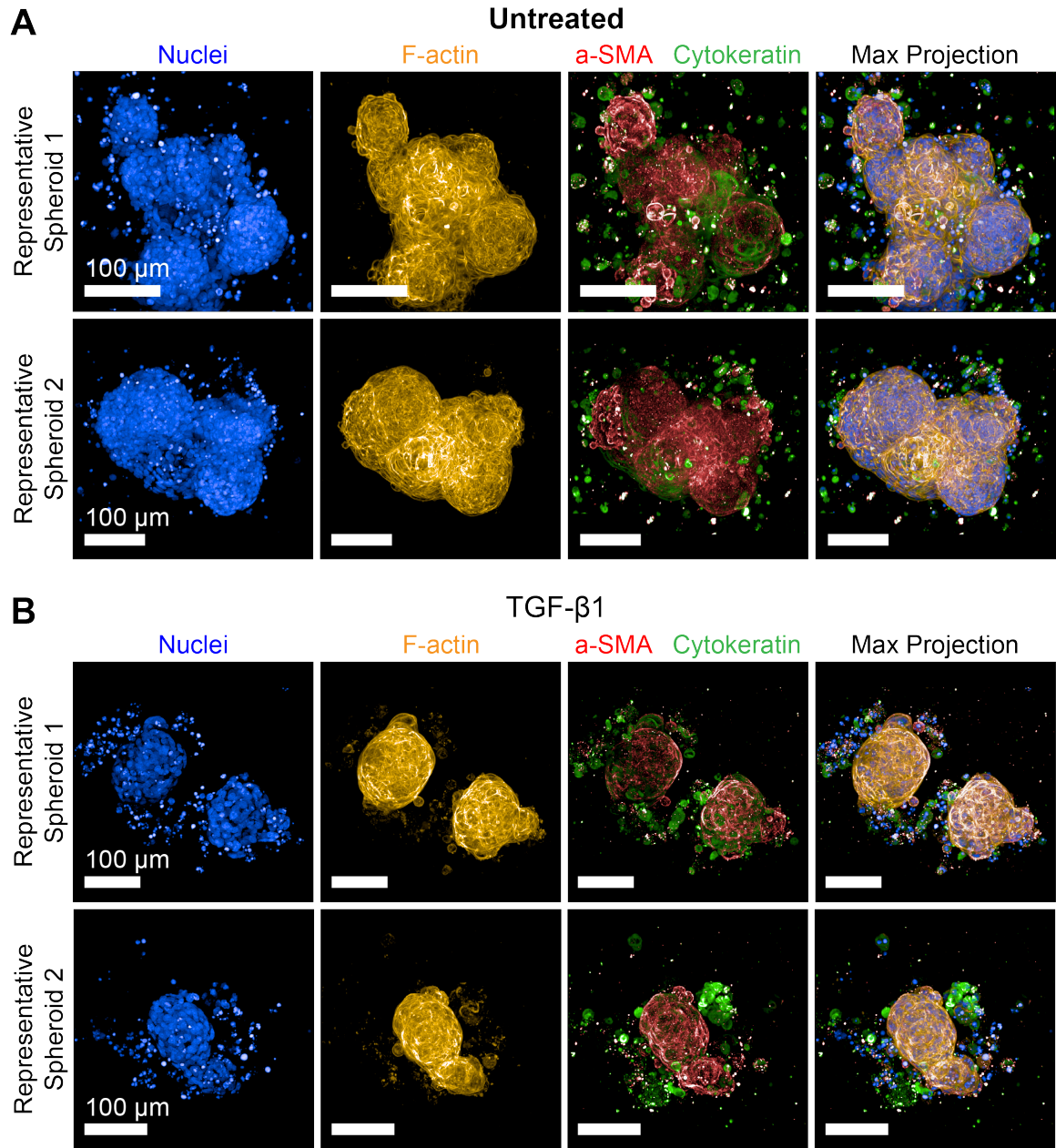


Figure 3.15: Characterizing Fibroblast and Epithelial Cell Coculture Microtissues. Fibroblast and epithelial cells were cocultured together in non-adherent agarose hydrogels and self-assemble into microtissues. (A) To represent 15-day untreated coculture morphometry and biomarkers, representative images show that microtissues sort and grow new microtissue lobes over time. The majority of the microtissue body is comprised of α -SMA myofibroblasts or stromal, myofibroblast-like epithelial cells. Epithelial cells positive for cytokeratin remain at the microtissue edge. (B) 15-day coculture microtissues exposed to 5 ng/mL TGF- β do not continue to grow or form lobes over time. Microtissues exhibit the same nuclear and actin morphologies and α -SMA and cytokeratin biomarker organization but with decreased microtissue growth.

dicative of nanomaterial aggregation within microtissues as initially hypothesized. TGF- β 1 exposed microtissues again exhibit decreased microtissue size after 15 days (Figure 3.13B), and are almost completely dead after 21 days (Figure 3.13C). 21-day untreated microtissues exhibit large ECM deposition regions, with gaps inside the microtissues that could be indicative of cell death (Figure 3.13C).

Changes in overall microtissue size were quantified and indicated decreased microtissue size as a result of carbon black, carbon nanotube, and G550 graphene nanosheet exposures (Figure 3.14). We hypothesized that this is due to increased compaction driven by cell-nanomaterial interactions within the microtissue, but could be due to decreased viability. Additional experiments were performed to evaluate microtissue stability over time due to the increase in untreated microtissue size observed after 10 days (Figure 3.14). Untreated microtissues exhibit internal proliferation over time, resulting in multi-lobed microtissue formation. These multi-lobed microtissues have wide, central regions that branch into each lobe and can reach 200 μ m in width by 10-days (Figure 3.15A). Alternatively, TGF- β 1-exposed microtissues exhibit decreased size and an abundance of cell debris in the microtissue periphery (Figure 3.15B). To incorporate TGF- β 1 exposure as a positive control to induce biomarkers of fibrosis, only microtissues before 7 days of age were evaluated for biomarker characterization after particle exposure.

3.4.5 Coculture microtissues are comprised of differentiated myofibroblasts and mesenchymal cells.

To characterize cell-sorting and the cell phenotypes inherent in the coculture microtissues, 5-day microtissues were fixed, stained with cytokeratin and α -SMA biomarkers, and evaluated for particle aggregation. Additionally, to better observe changes to the cell organization within microtissues, center sections were imaged for actin and nuclear organization. Representative images of (Figure 3.16A) untreated and (Fig-

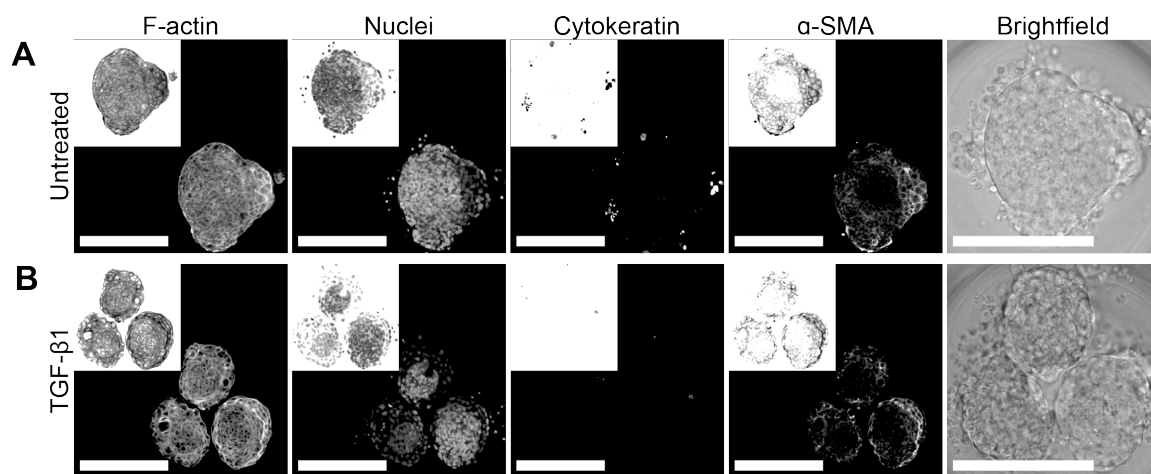


Figure 3.16: 5-day Coculture Microtissue Morphology and Biomarkers. 15-day microtissues show excessive microtissue growth not observed at timepoints below 7 days. 5-day (A) untreated and (B) TGF- β 1-exposed microtissues were characterized. Inverted and enlarged insets highlight actin and nuclear organization. (A) Untreated microtissues exhibit a compact and smooth microtissue body with tightly clustered epithelial cell nuclei. In contrast (B) TGF- β 1-exposed microtissues experience pronounced cell death, highlighted by cell debris surrounding the microtissue bodies, and are smaller in size. Cytokeratin-positive cells, localized to the microtissue exterior, are sparse, but most of the microtissue body is positive for α -SMA incorporated into the cell cytoskeleton. Inverted insets aid in viewing subtle organization changes within microtissues.

ure 3.16B) TGF- β 1-exposed microtissues exhibit comparable actin organization and compaction of cell types as indicated by the nuclear signal. Additionally, untreated microtissues have few cytokeratin-positive epithelial cells at the microtissue periphery, while TGF- β 1 exposures remove this population. Another peripheral population of α -SMA-positive fibroblasts or epithelial cells surround a central microtissue region negative for both phenotypic biomarkers. We hypothesize that the central cell population is comprised of mesenchymal-epithelial cells.

Furthermore, we characterized nanomaterial-mediated changes in coculture microtissue organization using the same morphological and biomarker endpoints. Carbon black (Figure 3.17A), asbestos (Figure 3.17C), and graphene sheets (Figure 3.17D, E) are incorporated into microtissues but do not exclude cells from peripheral regions

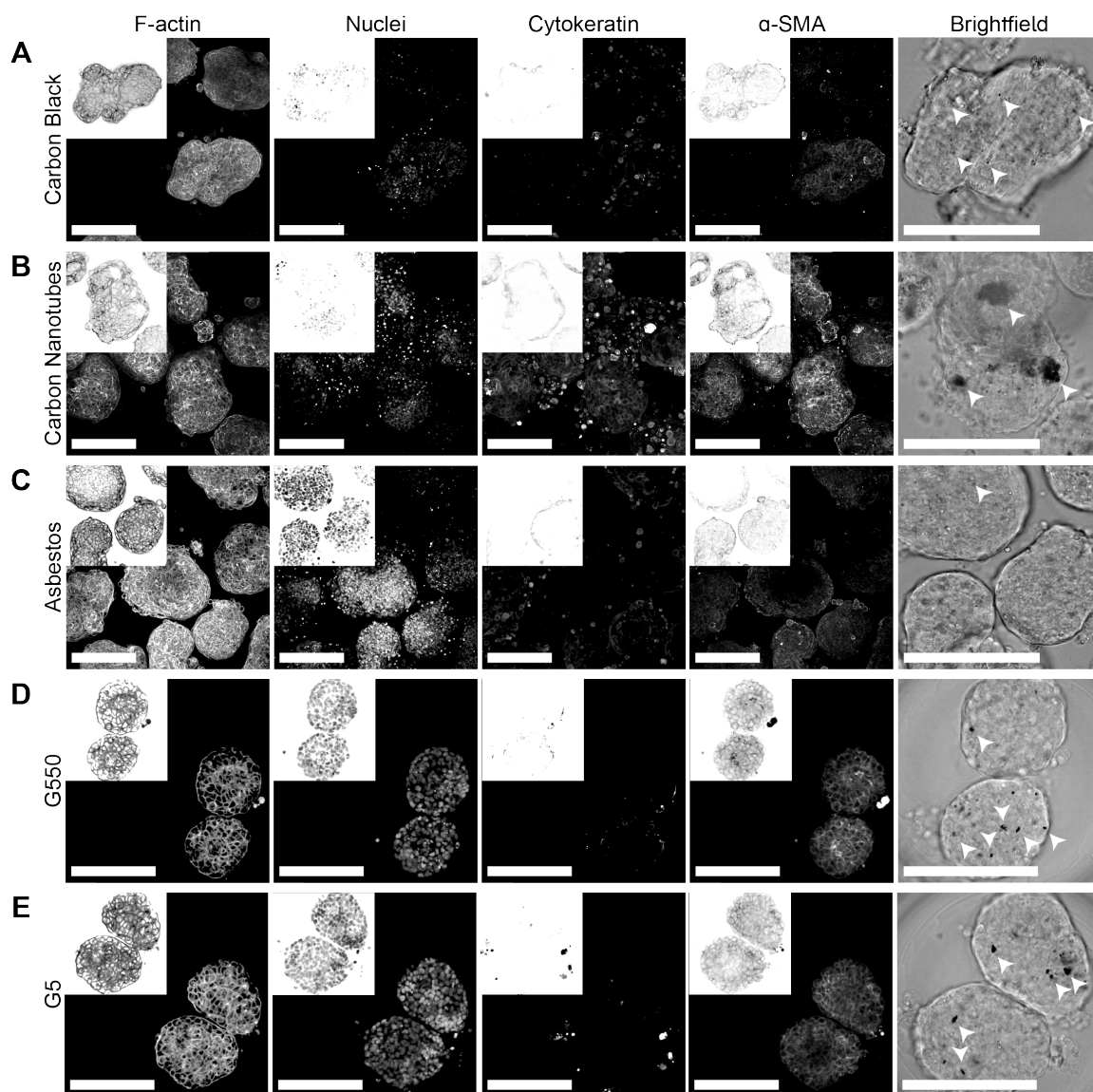


Figure 3.17: 5-day Coculture Microtissue Morphology, Biomarkers, and Particle Incorporation.

5-day microtissues exposed to (A) carbon black, (B) carbon nanotubes, (C) asbestos, (D) G550 graphene nano-sheets, and (E) G5 graphene micro-sheets were characterized. Inverted and enlarged insets highlight actin and nuclear organization. Cytokeratin-positive cells, localized to the microtissue exterior, are sparse, but most of the microtissue body is positive for α-SMA incorporated into the cell cytoskeleton. Enlarged brightfield images highlight organization and aggregation of nanomaterials (white arrows) within microtissues, with changes to actin organization only observed after carbon nanotube exposure. Inverted insets aid in viewing subtle organization changes within microtissues.

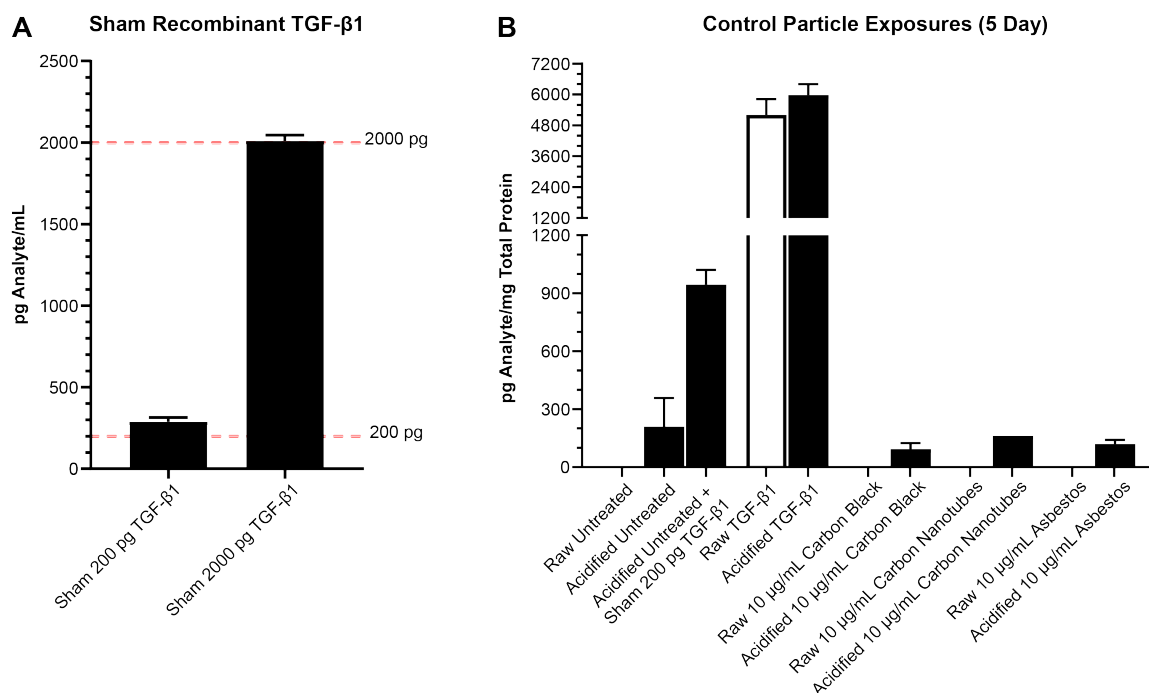


Figure 3.18: Free and Bound TGF- β 1 in Coculture Microtissues.

(A) Recombinant TGF- β 1 was spiked into Luminex analyte solution to validate Luminex quantification of TGF- β 1. Predicted values of sham TGF- β 1 are denoted using dotted red lines. (B) Exposure to control particles does not increase TGF- β 1 protein. Experiments were performed in duplicate.

around nanomaterial deposits. Alternatively, carbon nanotubes (Figure 3.17B) form larger aggregates of deposited material that exclude cells, as illustrated by an absence of actin in regions of carbon nanotube aggregates. It is unclear if this is due to low viability in these regions. Microtissues exposed to carbon nanotubes and asbestos exhibit slightly higher populations of epithelial cells at the microtissue periphery, and carbon nanotube and graphene exposures induce more α -SMA differentiation inside microtissues. The microtissue cores that are negative for both biomarkers are still present in each microtissue exposure.

3.4.6 Coculture microtissues do not up-regulate TGF- β 1 production after nanomaterial exposures.

Finally, we wanted to interrogate the potential for nanomaterial exposures to promote TGF- β 1 production. Luminex protein assays were benchmarked to sham concentrations of the same recombinant TGF- β 1 used in experimental conditions to support assay accuracy (Figure 3.18A). Live 5-day microtissues were collected and lysed to collect TGF- β protein inside microtissue cells or embedded in deposited matrix within microtissues. Acidification of sample lysates released bound TGF- β for detection. Even at high concentrations of nanomaterial exposure, carbon nanotube and asbestos do not significantly promote TGF β 1 production as hypothesized (Figure 3.18B). We hypothesize that this is likely due to the early model timepoint, supporting the need for a more stable, long-term model of fibroblast-epithelial cell coculture interactions to evaluate chronic exposure to the toxic, fibrous nanomaterials.

3.5 Conclusions and Future Directions

Fibroblast-only microtissues with and without collagen type 1 failed to generate models of fibroblastic foci that could be characterized for gene and protein expression. Fibroblast microtissues in the absence of collagen type 1 exhibited elevated levels of cell death. In contrast, the integration of collagen into fibroblast microtissues depleted samples of RNA and protein, rendering them inaccessible for evaluation by gene expression array or western blotting. While chromogenic or immunofluorescent staining techniques could evaluate alternatives to gene and protein expression, the absence of an additional assay to confirm immunofluorescent staining results limited the analytical capabilities of the model and decreased the model's potential use by other laboratories in academic and regulatory settings if published.

Fibroblast-only microtissues are comprised of a cell type that does not typically associate strongly with one another due to the absence of cell-cell junctions and the strong expression of integrins and focal adhesion proteins that result in preferential ECM-binding. Physiologically and functionally, any resulting cluster of associated fibroblasts forming a microtissue most closely resembles a pro-fibrotic fibroblastic focus even at its healthiest baseline. Even if IMR-90 fibroblasts did not differentiate in a non-adherent 3D microenvironment, the fibroblast-only microtissue model would be limited to questions associated with fibroblast to myofibroblast differentiation, perpetuation and maintenance of myofibroblast phenotypes, and ECM deposition and remodeling at acute and chronic endpoints. To increase the complexity and physiological relevance of a fibroblast-only microtissue, the addition of macrophages could potentially introduce a clearance mechanism and pro-fibrotic secreted factor crosstalk between macrophages and fibroblasts. In pro-fibrotic microenvironments, the influx of recruited macrophages, differentiation of resident macrophages, and the activation and differentiation of fibroblasts as a result of macrophage activation produces a granuloma-like cluster of cells that perpetuates activation of the TGF- β signaling pathway. By mixing single-cell suspensions of macrophages and fibroblasts, we hypothesized that the resulting microtissue would resemble a granuloma with epithelioid macrophages and fibroblasts and myofibroblasts. The evaluation of this type of interaction is just now being newly evaluated to include direct physical interactions between macrophages and fibroblasts. Despite initial data on IMR-90 fibroblast and THP-1 macrophage coculture microtissues, the inclusion of an alternative primary, hTERT immortalized human lung cell line introduces another factor that cannot be compared to another of our other existing microtissue platforms or the published literature. Additionally, evaluation of the effects of hTERT immortalization to drive the phenotypes that we observed was incomplete.

Several additional experiments are required to evaluate the fibroblast-macrophage microtissue model. Future experimental work should evaluate macrophage lysosomal integrity and cytokine profiles associated with graphene exposures. Primary fibroblasts should be evaluated for lysosomal integrity and particle endocytosis. Coculture microtissue morphology should be evaluated for all particle exposures with a recombinant TGF- β 1-exposed control for myofibroblast differentiation. Additional morphological evaluations and high-resolution images of α -SMA and cadherin-11 biomarkers, alongside particle incorporation, are required to evaluate if minor internal changes occur that are not quantified through Luminex quantification of TGF- β 1 production and secretion. If internal regions of microtissues are positive for myofibroblast differentiation and cadherin-11 interactions, higher resolution RNA in-situ hybridization or immunohistochemistry on central microtissue sections could observe single-cell changes in gene expression and altered physical interactions between cadherin-11 and β -catenin and differential production of TGF- β 1. Finally, Luminex collection profiles should be completed and include an additional combination TGF- β RI inhibitor and recombinant TGF- β 1 treatment as an experimental control in monoculture and coculture microtissues. Our knowledge of this type of fibroblast-macrophage microtissue model is currently based on one newly published paper and our preliminary research, and this model most importantly requires further morphological analysis, imaging of cadherin-11 organization within microtissues between cell types, and longevity testing for viability with and without nanomaterial exposures, among many other experiments.

Ideally, higher-level future research would focus on evaluating the extent of the normal gene and protein expression in the primary lung fibroblast line compared to primary human lung fibroblasts. Quantification of gene and protein expression to compare baseline fibroblast function would identify similarly expressed pathways and target questions that could be accurately evaluated using the primary cell line. Fu-

ture research should also evaluate changes from baseline fibroblast and macrophage gene expression in coculture conditions, which could also be compared to published healthy and fibrotic gene expression and proteomics datasets with limited comparability. More advanced research into cell type ratios (data not shown) and pretreatments to differentiate and activate macrophages that could better match the baseline microtissue to healthy and fibrotic lung gene and protein expression could be completed. At present, cell type ratios were optimized to promote microtissue formation and stability and quantify the presence of cell-cell interactions driven by cadherin-11, cytoskeletal organization, and nanomaterial exposure. While these primary inspirations for experimental conditions would allow us to evaluate our specific experimental question, these approaches may not be the most efficient for producing a translationally-relevant model to evaluate the pro-fibrotic potential of nanomaterials to drive changes to physical cell-cell interactions. Finally, further work to evaluate the specificity of currently available cadherin-11 antibodies and their localization in microtissues with and without nanomaterial exposure is required to assess the value of this model to identify pro-fibrotic nanomaterials that drive changes in physical cell-cell interactions.

Goals for microtissue stability included viability, proliferation control, conservation of undifferentiated cell culture phenotypes, and the ability to grow microtissues out to chronic timepoints with both untreated and TGF- β -exposed control microtissues. By our definition, the most stable and successful model developed to address the third aim of this thesis work was the macrophage and fibroblast coculture microtissue model. The success of the fibroblast-macrophage microtissue model supports the incorporation of primary cell lines instead of IMR-90 fibroblasts. Primary cell incorporation may improve model stability and efficacy to allow for research of patient-to-patient heterogeneity in models and double as nanomaterial screening tools. Alternatively, non-adherent or adherent air-liquid interface models of bron-

cholar architectures offer models that still incorporate the cell-cell interactions that we wished to interrogate. However, air-liquid interface models also require extreme benchmarking efforts required to reproduce morphological and molecular endpoints that are currently inconsistent between laboratories. Finally, fibroblast and epithelial cell coculture microtissues are viable with TGF- β 1 positive-controls before 7 days and exhibit proliferation and active sorting activity throughout the lifetime of the microtissue. As a result of fibroblast and epithelial cell differentiation within the microtissue, there is no baseline, healthy microtissue that models homeostatic human lung cell interactions.

IMR-90 fibroblast and BEAS-2B epithelial cell coculture microtissues concurrently exhibited valuable and detrimental features that solidify its decreased potential as a translationally-relevant and chronic endpoint model. While the model exhibited the best cell-cell interaction, compaction, and viability, the model uncontrollably proliferates even in less complex media with fewer growth factors. Furthermore, the decrease in the long-term viability of what should be a TGF- β -exposed positive control condition and increased myofibroblast and mesenchymal differentiation observed in organized microtissues suggests poor recapitulation of healthy lung cell-cell interactions. While we knew that the cell ratio would not recapitulate healthy lung tissue ratios between epithelial cells and fibroblasts, we did not hypothesize differentiation in the epithelial cell population. The mix of the resulting mesenchymal populations could evaluate re-epithelialization in the final phase of wound healing. In the final phases of successful wound healing, newly differentiated mesenchymal-epithelial cells migrate to sites of newly deposited ECM, repopulating the repaired basement membrane, and de-differentiating to fulfill their role as the new pulmonary epithelium. As such, the use of this model is limited in the number of research questions that can be evaluated. However, the model could be further characterized to evaluate drivers

of de-differentiation as a drug-testing platform; however, this is currently beyond the scope of this research.

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3.7 Supplementary Information

Weight or Vol. Calc. (50mL)	Scale Clearing Components
Sorbitol (g)	20.0
Glycerol (g)	5.0
Urea (g) (60.056 g/mol)	12.0
Triton X-100 (g)	0.1
DMSO (mL)	7.5-12.5
PBS (mL)	fill to 50

Table 3.1: Scale Clearing Solution Components

Chapter 4

Conclusion and Closing Statements

The research described herein has been facilitated by recent advances in image analysis and biostatistics. Historically, the field of toxicology has been impeded by the inability to quantify sub-cytotoxic changes, resulting in the need to evaluate high concentrations of potential toxicants to detect toxicity. Toxicologists have known, in part, and are still realizing the extent to which the world-wide human population and aquatic environments are being exposed to countless environmental contaminants. As a result, humans are routinely and, in some cases, permanently exposed to several toxicants at a time, resulting in co-exposure, transient, and adaptive effects that can be subtle. Application of image analysis and biostatistics will lead toxicology into the 21st century, where we will better define the effects of toxicants at concentrations relevant to human exposure and become capable of measuring, not just predicting, co-exposure, repeated exposure, and low-dose exposure effects using molecular and morphological biological features. Many molecular and morphological features have the tools needed to evaluate them through sample labeling and imaging, with several case studies and in vivo and in vitro work that allows us to benchmark the overall utility of our models. Overall, we are close to being able to accurately detect changes in small percentages of cell populations that can drive chronic disease. We can detect

those changes at acute timepoints and extrapolate based on benchmarks, decreasing the cost and time of research, potentially decreasing animal use, and informing how we remediate toxicant release. We can also detect and measure toxicant exposures and assess the efficacy of prophylactic or clinical treatments. In the context of this research, we could use these tools to inform safety-by-design initiatives to make nanomaterial exposure safer while preserving functional properties.

For the ease of communication with regulatory agencies, some toxicologists are moving toward the development of Adverse Outcome Pathways (AOPs). AOP flow charts for toxicity testing detail the mechanisms of disease pathogenesis, including an initiating event, biomarkers that change during disease pathogenesis and indicate damage or transformation, and pathological traits or symptoms. Pairing image and multivariable analysis with other single-cell approaches adds value to existing in vitro assay regulatory paradigms, especially where existing AOPs offer targeted biomarkers for study. Aims 1 and 2 of this thesis work utilized primary, IMR-90 lung fibroblasts to evaluate the effects of substrate and nanomaterial exposure to drive fibroblast to myofibroblast differentiation at acute timepoints. We confirmed several effects of cell culture on soft substrates that have been previously observed but also identified effects of nanomaterial exposure that could lead to chronic myofibroblast differentiation apart from the complex cell-cell, cell-ECM, and inflammatory signaling associated with the pro-fibrotic microenvironment. If direct exposure to deposited toxic nanomaterials alone is capable of triggering pro-fibrotic myofibroblast differentiation, minimal interaction with deposited nanomaterials could trigger the progressive disease. Future work for graduate students could evaluate the mechanical stimuli associated with myofibroblast activation that is currently poorly described in the literature. Additionally, identifying shortcomings of evaluating biopersistent nanomaterial exposures on common, traditional cell culture platforms suggests a need

for a paradigm shift in the way that toxicologists evaluate cell phenotype changes and perform toxicity testing overall or for specific types of toxicants.

By performing image analysis using TGF- β signaling endpoints, we observed that α -SMA was the most sensitive endpoint. α -SMA was able to detect myofibroblast differentiation across stiff and soft substrates that we failed to observe using the p-Smad2/3 biomarker. While α -SMA was capable of identifying sub-populations of differentiated cells on rigid and soft substrates, the variability in percent population differentiation between wells and plates made limited our capability to identify statistically significant changes in populations associated with TGF- β or nanomaterial exposures. Through the development of the analysis pipeline for feature extraction in perinuclear regions of fibroblasts, we were able to bypass needing to identify then entire cell body and performed feature extraction to generate feature profiles across substrates and nanomaterial exposures. We relied on confidence intervals and FAMD principal component analysis to visualize subtle differences of feature profiles driven by subtle sub-population changes. Conventional approaches to principal component analysis use plotting of confidence intervals to evaluate the complete separation of clusters that denotes different phenotypic profiles. We utilized this technique to identify changes in sub-population differentiation and observed increases in population heterogeneity through an increased confidence interval size.

Additionally, we observed the transformation of confidence intervals away from untreated and TGF- β 1 controls. Finally, we utilized hierarchical clustering to identify overall similarity in exposure profiles on stiff and soft substrates, observing that soft substrates allow cells to recognize fibrous carbon nanotubes and recognize sheet-like nanomaterials. The value in this technique lies in its potential for universal application across sample types and pathologies in the absence of complete single-cell segmentation, which is only possible for spatially separated cells with cleanly labeled membrane targets. Furthermore, the success of this analysis approach also

lends itself to translating the analysis for 3D in vitro platforms. Application of this analysis framework could aid in evaluating more complex phenotypic changes driven by cell-cell interactions, coculture and multi-cell culture, and phenotype transience or adaptation at acute or chronic timepoints currently unavailable for analysis using traditional cell culture.

Aim 3 attempted to identify indicators of fibroblast to myofibroblast differentiation in a microenvironment that is more complex and more similar to the lung microenvironment. The creation of several 3D, non-adherent microenvironments in which fibroblasts interact and can coexist with nanomaterials offered opportunities to evaluate more chronic timepoints. However, each 3D model exhibited limitations that restricted the breadth of scientific questions that could be evaluated. Goals for microtissue stability included viability, proliferation control, conservation of undifferentiated cell culture phenotypes, and the ability to grow microtissues out to chronic timepoints with both untreated and TGF- β -exposed control conditions. By our definition, the most stable and successful model developed to address the third aim of this thesis work was the fibroblast and macrophage coculture microtissue model. The success of the fibroblast and macrophage coculture microtissue model supports the incorporation of primary cell lines instead of IMR-90 fibroblasts. Primary cell incorporation may improve model stability and efficacy to allow for research on patient-to-patient heterogeneity and function as a nanomaterial screening tool. Alternatively, non-adherent or adherent air-liquid interface models of bronchiolar architectures offer models that still incorporate the cell-cell interactions that we wished to interrogate. However, air-liquid interface models also require careful benchmarking efforts to reproduce morphological and molecular endpoints that are currently inconsistent between laboratories. Regarding fibroblast and macrophage coculture microtissues, several additional experiments are required to evaluate the fibroblast and macrophage coculture microtissue model, making it a potential, extensive thesis

project. Future experimental work should evaluate macrophage lysosomal integrity and cytokine profiles associated with graphene exposures. Primary fibroblasts should be evaluated for lysosomal integrity and particle endocytosis. Coculture microtissue morphology should be evaluated for all particle exposures with a recombinant TGF- β 1-exposed control for myofibroblast differentiation. Additional morphological evaluations and high-resolution images of α -SMA and cadherin-11 biomarkers, alongside particle incorporation, are required to evaluate if minor internal changes occur that are not quantified through Luminex quantification of TGF- β 1 production and secretion. If internal regions of microtissues are positive for myofibroblast differentiation and cadherin-11 interactions, higher resolution RNA in-situ hybridization or immunohistochemistry on central microtissue sections could observe single-cell changes in gene expression and altered physical interactions between cadherin-11 and β -catenin and differential production of TGF- β 1. Finally, Luminex collection profiles should be completed and include an additional combination TGF- β RI inhibitor and recombinant TGF- β 1 treatment as an experimental control in monoculture and coculture microtissues. Our knowledge of this type of fibroblast-macrophage microtissue model is currently based on one newly published paper and our preliminary research. This model requires further morphological analysis, imaging of cadherin-11 organization within microtissues between cell types, and longevity testing for viability with and without nanomaterial exposures, among many other experiments.

Ideally, higher-level future research would focus on evaluating the extent of the normal gene and protein expression in the primary, immortalized lung fibroblast cell line compared to primary human lung fibroblasts. Quantification of gene and protein expression to compare baseline fibroblast function would identify similarly expressed pathways and target questions that could be accurately evaluated using the primary cell line. Future research should also evaluate changes from baseline fibroblast and macrophage gene expression in coculture conditions, which could also be compared to

published healthy and fibrotic gene expression and proteomics datasets with limited comparability. More advanced research into cell type ratios (data not shown) and pretreatments to differentiate and activate macrophages that could better match the baseline microtissue to healthy and fibrotic lung gene and protein expression could be completed. At present, cell type ratios were optimized to promote microtissue formation and stability and quantify the presence of cell-cell interactions driven by cadherin-11, cytoskeletal organization, and nanomaterial exposure. While these primary inspirations for experimental conditions would allow us to evaluate our specific experimental question, these approaches may not be the most efficient for producing a translationally-relevant model to evaluate the pro-fibrotic potential of nanomaterials to drive changes to physical cell-cell interactions. Finally, further work to evaluate the specificity of currently available cadherin-11 antibodies and their localization in microtissues with and without nanomaterial exposure is required to assess the value of this model to identify pro-fibrotic nanomaterials that drive changes in physical cell-cell interactions.

IMR-90 fibroblast and BEAS-2B epithelial cell coculture microtissues concurrently exhibited advantageous and disadvantageous features as a translationally-relevant and chronic endpoint model. Although the model exhibited the best cell-cell interaction, compaction, and viability, the spheroid uncontrollably proliferates even in simplified media with reduced growth factors. Furthermore, the decrease in the long-term viability of what should be a TGF- β -exposed positive control condition and increased myofibroblast and mesenchymal differentiation observed in organized microtissues suggests poor recapitulation of healthy lung cell-cell interactions. As a result of fibroblast and epithelial cell differentiation within the microtissue, there is no baseline, healthy microtissue that models homeostatic human lung cell interactions. Although we recognized that the cell ratio would not recapitulate healthy lung tissue ratios between epithelial cells and fibroblasts, we did not hypothesize differentiation in the

epithelial cell population. The mix of the resulting mesenchymal populations could evaluate re-epithelialization in the final phase of wound healing. In the final phases of successful wound healing, newly differentiated mesenchymal-epithelial cells migrate to sites of newly deposited ECM, repopulating the repaired basement membrane, and de-differentiating to fulfill their role as the new pulmonary epithelium. As such, the use of this model is limited in the number of research questions that can be evaluated. The fibroblast and epithelial cell coculture model could be further characterized to evaluate drivers of de-differentiation as a drug-testing platform; however, this is currently beyond the scope of this research.

Several future projects could build on our 3D research, including completing characterization of a model for a specific set of scientific questions in the context of the human lung, evaluating the efficacy of these models for toxicant and drug testing where each model may be able to detect chemical exposures but not nanomaterial exposures, and further development of a model with all three key cell type components that drive inflammation, clearance, and ECM remodeling. Furthermore, application of the techniques utilized to evaluate fibroblast and myofibroblast populations in Aims 1 and 2 could be applied to measure other phenotypic changes in matching macrophage and epithelial cell populations utilized in our 3D models for comparison. Quantification of each cell type's response to biopersistent nanomaterial exposure at low concentrations could make the application of in vitro cell culture more useful in the absence of more complex air-liquid interface or 3D assays, again offering several opportunities to further this research. The breadth of potential future research spans several scientific disciplines and analysis techniques and offers several avenues for expansion upon this thesis work that could significantly contribute new toxicity testing paradigms.