The Particulars of Particulate Matter Induced Myofibroblast Differentiation

by

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Abstract

Particulate matter (PM) has long been appreciated as a biologically significant component of air pollution that has major impacts on public health. Exposure to PM less than 2.5 μm in diameter ($PM_{2.5}$) is associated with mortality, and increased incidence of lung diseases. While the effect that $PM_{2.5}$ exposure has on many cell types, such as epithelial cells, is relatively well studied, the effect it has on mesenchymal cells, such as fibroblasts, is less understood. This study seeks to determine the effect that $PM_{2.5}$ exposure has on fibroblast-to-myofibroblast differentiation, which is a critical step in pulmonary fibrosis, a disease that has been shown to be exacerbated by $PM_{2.5}$ exposure.

We assessed the effect that $PM_{2.5}$ exposure has on fibroblast biology and myofibroblast differentiation through four research aims. In Aim 1, we delineated the conditions and determinants by which ambient $PM_{2.5}$ affected fibroblast-to-myofibroblast differentiation and found that, interestingly, repeated low concentration $PM_{2.5}$ exposures promoted increases in αSMA and collagen, markers of myofibroblast differentiation. Furthermore, follow-up studies utilizing pharmacological inhibitors showed that NF-κB is critical for this effect.

In Aim 2, we sought to investigate the effect that $PM_{2.5}$ exposure has on bone morphogenic protein (BMP)2 expression and secretion in fibroblasts. BMP2 is a cytokine that can promote or inhibit myofibroblast differentiation and can be activated by NF-κB. We found that $PM_{2.5}$ exposure promoted a dose-dependent increase in BMP2 transcript and secreted

protein. The role of BMP2 on $PM_{2.5}$-induced increase in myofibroblast differentiation was less clear; however, as treatment with exogenous BMP2 promoted myofibroblast differentiation, while blocking endogenous BMP2 with an siRNA or inhibitory protein (noggin), also promoted myofibroblast differentiation. This implies that a certain level of BMP2 signaling is critical for fibroblasts to maintain homeostasis, but that too much signaling has deleterious effects as well.

In Aim 3, we examine the effect that $PM_{2.5}$ exposure has on cytokine secretion and DNA methylation in lung fibroblasts. We analyze fibroblasts exposed to $PM_{2.5}$ on a DNA methylation array, as well as supernatants collected from similarly exposed fibroblasts on a Luminex cytokine kit. We found that low concentrations of $PM_{2.5}$ promoted methylation changes in genes enriched in cell cycle whereas high concentrations promoted methylation changes in a completely different set of genes, including those enriched in adhesion, neuronogeneis, and cell signaling. Additionally, IL-12 was found to be significantly upregulated following low concentration exposure to $PM_{2.5}$ in our Luminex experiments.

We sought to determine if $PM_{2.5}$ exposure *in vivo* increases susceptibility to pulmonary fibrosis in Aim 4. The data in our prior Aims suggest that $PM_{2.5}$ exposure promotes myofibroblast differentiation, an important process in fibrogenesis. Here, we exposed mice to $PM_{2.5}$ and assess their susceptibility to bleomycin-induced fibrosis. Despite promising results in a pilot experiment, where we demonstrated a trend toward higher collagen content, as measured by hydroxyproline, in the lungs of male mice exposed to $PM_{2.5}$ prior to bleomycin treatment, we were unable to replicate the results in a larger study with female mice. This implies that sex may have significant effects on susceptibility to $PM_{2.5}$ exposure.

$PM_{2.5}$ exposure results in a myriad of changes in fibroblast biology, changes that can often promote fibrosis. The exact effects of $PM_{2.5}$ on fibroblasts vary depending on the duration,

frequency, and concentration of exposure, demonstrating the public health significance of even low levels of air pollution.

Chapter 1 Particulate Matter Exposure and its Effects on Respiratory Health

1.1 Introduction

Particulate matter (PM), defined as the sum of all solid and liquid suspended in the atmosphere, is one component of air pollution that has been shown to cause significant worldwide mortality, contributing to an estimated 4.14 million worldwide deaths per year[1, 2]. Both the United States Environmental Protection Agency (EPA) and the World Health Organization (WHO) classify PM based on aerodynamic diameter, a measure of size that accounts for the particles irregular shape; with PM_{10} referring to particles < 10 μm in diameter, and $PM_{2.5}$ referring to particles < 2.5 μm in diameter[3, 4]. Smaller particles are considered more harmful and impose a greater risk to human health due to their ability to penetrate deeper into the lung, and even enter the bloodstream, where they contribute to systemic-related diseases. Smaller particles also have a higher surface area to mass ratio, which increases their chemical reactivity, and they possess the ability to remain suspended in the atmosphere for longer durations[5].

PM is derived from natural (dust storms, ocean spray) and anthropogenic (fossil fuel consumption, industrial processes) sources, with artificial sources being more relevant to health outcomes. This is due to the composition of the particles produced by artificial sources, and the relative proximity of anthropomorphic emission sources to human activity. This is shown in several studies examining the relative contribution of individual PM components to overall PM toxicity. Broadly speaking, PM generated by vehicle and diesel emissions are high in elemental carbon and organic carbon matter, as well as the metals nickel and vanadium.

Exposure to PM with this compositional profile was found to result in increased emergency room visits and hospitalizations due to cardiovascular and pulmonary complications, as well as increased overall mortality[6, 7]. Conversely, exposure to PM with a compositional profile higher in silica and crustal minerals, indicative of natural PM formation, was found to have more modest effects on hospitalization and mortality[6, 7, 8]. Furthermore, the effects of $PM_{2.5}$ exposure have also been shown to vary based on source location[9, 10]. Here, we chose to focus on ambient PM collected from multiple urban environments, and specifically $PM_{2.5}$ due to its ability to penetrate deep into the lungs and affect the lower lung parenchyma.

1.2 The Role of Particulate Matter in Human Health

It is well established that ambient $PM_{2.5}$ exposure has significant and deadly health effects. Exposure to $PM_{2.5}$ has been associated with numerous diseases, including cardiovascular disease[11], atherosclerosis[12], stroke[13], acute exacerbations of chronic obstructive pulmonary disease (COPD)[14, 15, 16], lung cancer[17, 18, 19, 20], pulmonary infections[4, 21], and asthma[22, 23]. Various studies have found that every 10 $\mu g/m^3$ increase in ambient $PM_{2.5}$ concentration correlates to a 0.23% increase in deaths due to stroke[13], a 3.1% increase in COPD-related emergency room visits[16], and a 1.8% increase in pneumonia incidence among children[21].

Over the years, various attempts have been made to curb the effects of air pollution. While this has helped to decrease ozone and indoor $PM_{2.5}$ levels, which were previously recognized as greater contributors to disease than outdoor $PM_{2.5}$, the improvements in morbidity and mortality from decreases in ozone and indoor $PM_{2.5}$ were not met with the same improvements from ambient or outdoor $PM_{2.5}$[1]. This is despite stricter regulation and an overall decrease in levels of ambient $PM_{2.5}$, especially in developed countries. In fact, although indoor air pollution used to be the main driver of mortality in the past, outdoor $PM_{2.5}$ now unfortunately

kills nearly twice as many people as indoor $PM_{2.5}$ and over six times as many people as ground level ozone. The improvements in mortality from ozone and indoor air pollution from 1990-2017 are 3 to 5-fold greater than the improvements made from curbing outdoor $PM_{2.5}$[1]. These data indicate the importance of limiting not just indoor, but outdoor $PM_{2.5}$ as well, and how even low levels of ambient $PM_{2.5}$ can still exert significant morbidity and mortality[1]. Recent studies have indicated that low levels of $PM_{2.5}$, even levels below the standards set by many countries, can have a significant impact on health. Reduced pulmonary function[24], lower birth weight[25], and increased risk of hospitalization and death[26, 27] have all been linked to $PM_{2.5}$ exposures at or even below the current $PM_{2.5}$ US National Ambient Air Quality Standards. The mechanism(s) by which these low levels of $PM_{2.5}$ exposure contribute to negative health outcomes is not completely known.

1.3 PM Effects in the Airway

The lung fulfills the critical role of facilitating gas exchange and total body respiration. However, in fulfilling this role, it leaves itself open to inhaled environmental insults that may promote pathogenesis. The lungs communicate with the environment through progressively smaller respiratory tracts that extend from the oral and nasal orifice to the terminal bronchioles. Defenses along the airway help protect the lung from environmental insults, but the airways are also susceptible to damage and toxicity themselves. While the oral and nasal portions of the respiratory tract protect the airway through mucus production and the ciliary action of nasal hairs, they are still susceptible to damage by PM exposure. Janssen *et al*. (2014) and Hong *et al*. (2016) showed that exposure to PM resulted in nasal inflammation and increased ROS levels in nasal epithelial cells[28, 29].

The trachea and large bronchi utilize cilia and mucus to capture large particulates and sweep them to the oropharynx. Exposure to particulates cause increases in ROS, and the release of IL-6 and IL-8 in bronchial epithelium[30, 31]. PM also disrupts the cytoskeletal regulation, cellular proliferation and cellular differentiation of bronchial epithelial cells[8] and activates DNA damage responses[32]. Despite the presence of cilia and mucus production along the airways that filter larger particulates, $PM_{2.5}$ still can travel down to the alveolar space where gas exchange occurs.

1.4 Effects of $PM_{2.5}$ in the lung parenchyma

As opposed to the airway, where mucus production and cilia play a larger role in host defense, the alveoli are the main distal gas-exchanging units of the lung and are protected from environmental insults by cells within the alveolar space, including alveolar epithelial cells and resident alveolar macrophages (AM). AMs phagocytose foreign particles. Although $PM_{2.5}$ can be cleared by phagocytosis from AMs[33], the insoluble nature of $PM_{2.5}$ often allow it to persist once it arrives in the alveolar space, and more soluble components of air pollution can often diffuse through the epithelial layer and even into the interstitium. This allows the harmful effects of $PM_{2.5}$ to often persist and contribute to chronic and long-term damage[34].

Studies on $PM_{2.5}$ have shown that $PM_{2.5}$ increases ROS and pro-inflammatory cytokine generation in the alveolar space[35], distorts alveoli formation during lung development[36], bypasses alveolar epithelial barrier function[37, 38], reduces immune response by increasing oxidative stress and mitochondrial damage in AMs[33, 39, 40, 41], and disrupts barrier function in endothelial cells via increase in vascular endothelial growth factor (VEGF) A secretion[42]. In alveolar epithelial cells, which are the main cells that line the alveolar space, PM has been shown to increase ROS levels,

induce the release of proinflammatory cytokines (IL-6, IL-8, and CCL2), activate TGFβ, increase cell contractility, and promote EMT[35, 43, 44, 45, 46].

1.5 PM and fibrotic lung disease

Epidemiologic studies have shown that exposure to PM is associated with the development and progression of fibrotic lung disorders, in particular, idiopathic pulmonary fibrosis (IPF). IPF is a chronic respiratory disease with no effective treatment. The disease is ultimately fatal with 5-year survival rates estimated to be 20-40%[47]. PM has been shown to contribute to acute exacerbations of IPF[48, 49], enhanced mortality due to IPF[49], and accelerated lung function decline in IPF patients[50]. To date there has only been one study that has shown a relationship between ambient PM exposure and incidence of IPF. Sack *et al.* (2017) found that exposure to $PM_{2.5}$ correlated with progression of markers of subclinical interstitial lung disease (ILD), suggesting that $PM_{2.5}$ exposure increases risk of developing clinically significant ILDs, such as IPF[51]. Other case-control studies found that exposure to metal dust[52, 53, 54, 55], silica[52], wood dust[52, 55], and agricultural dust[55] also increases an individual's risk of developing IPF.

Fibroblasts and activated myofibroblasts are believed to be central drivers of fibrosis progression. Fibroblasts are the major effector cells of IPF, responsible for the excessive deposition of extracellular matrix that is characteristic of the disease[56, 57]. Although epidemiologic literature suggests that $PM_{2.5}$ contributes to the development and progression of IPF, the mechanism by which this occurs is not known.

1.6 Effects of PM on fibroblast biology

While the effects of PM are well studied in bronchial and alveolar epithelial cells, their effects are less characterized in cells that inhabit the interstitium, such as pulmonary fibroblasts. One could argue that fibroblasts are protected from direct PM exposure by the epithelial barrier.

However, other studies have shown that exposure to $PM_{2.5}$ disrupts barrier function in alveolar epithelial cells, as measured by decreased levels of transepithelial electrical resistance[59, 60]. Accumulations of $PM_{2.5}$ have also been detected in the liver, indicating that $PM_{2.5}$ is able to bypass the epithelial barrier and enter circulation via the lung vasculature[37, 38]. This indicates that $PM_{2.5}$ can permeate the epithelial barrier, enter the lung interstitium and have direct effects on cells such as fibroblasts. Since lung fibroblasts maintain lung architecture by synthesizing extracellular matrix and participate in injury and repair, alterations in fibroblast function by $PM_{2.5}$ may have important implications where fibroblast play a significant role, such as in initiating repair after lung injury, or in diseases such as pulmonary fibrosis.

Prior studies have provided insight into the potential effects of PM exposure on fibroblast biology. Exposure to silica particles has been shown to cause endoplasmic reticulum stress, elevated collagen levels, cellular migration and proliferation[61], as well as ROS-mediated autophagy[62]. Exposure to ambient $PM_{2.5}$ causes mitochondrial dysfunction[63, 64], disrupts cellular metabolism[64], activates NF-κB[65], induces ROS production[66], and increases in proinflammatory cytokines[67] in fibroblasts. Finally, Xu *et al*. (2019) found that exposing fibroblasts directly to $PM_{2.5}$ results in activation of the TGF-β1/SMAD3 pathway, TGF-β1 excretion, and myofibroblast differentiation[46]. The results of these studies are summarized in Table 1.

While these studies provide insight into the effects of $PM_{2.5}$ on fibroblasts, they are often limited in that they utilize a single source of $PM_{2.5}$ and treat cells with a narrow range of $PM_{2.5}$ concentrations for a short duration. However, given that even low levels of $PM_{2.5}$ and chronic exposure are associated with the development of a myriad of diseases, it is conceivable that the effects of $PM_{2.5}$ on fibroblasts may differ or even be more extensive when examined in models of chronic exposure. Furthermore, previous work has shown that particulate matter from different

sources can have different biologic effects, demonstrating the utility of testing $PM_{2.5}$ from multiple sources[6, 7]. Here, our study sought to analyze the impact that longer, repeated exposures to $PM_{2.5}$ from multiple sources had on lung fibroblasts. We specifically examined the ability of $PM_{2.5}$ to promote myofibroblast differentiation and its effects on the secretion of bone morphogenic protein (BMP) 2. Myofibroblast differentiation, a process in which fibroblasts become aberrantly activated, is a central driver of fibrosis progression, and an important mechanism in IPF.

BMP2 is a secreted cytokine in the TGFβ superfamily of proteins[68]. As its name implies it is a potent inducer of bone and cartilage, however, it has also been shown to cause differentiation in a variety of cell types and tissues[68, 69, 70]. In certain contexts, BMP2 has also been shown to promote the differentiation of renal mesenchymal progenitor cells into myofibroblasts[71] and increase the expression of fibronectin, collagen I, and αSMA in mouse kidney cells[72]. Given these findings, we hypothesize that BMP2 may play a critical role in the mechanism by which $PM_{2.5}$ induces myofibroblast differentiation. Given that $PM_{2.5}$ may have pleiotropic effects on fibroblasts and the mechanism of action in these cells is not well-understood, we sought to better characterize the effects of $PM_{2.5}$ on fibroblasts, particularly its ability to affect myofibroblast differentiation.

1.7 $PM_{2.5}$ Mechanism of Action

There are many signaling pathways that have been shown to be triggered by $PM_{2.5}$. $PM_{2.5}$ is a potent inducer of reactive oxygen species (ROS) and oxidative stress, which in turn induces a variety of inflammatory responses, including activation of nuclear factor κB (NF-κB), nuclear factor (erythroid-derived 2)-like 2 (Nrf2), and c-Jun kinase (JNK)[73]. Activation of these pathways results gene expression changes that affect cell morphology and function. $PM_{2.5}$ is rich

in aromatic hydrocarbons and other xenobiotic chemicals that can activate the aryl hydrocarbon receptor (AhR). Studies examining the effect of $PM_{2.5}$ exposure in a variety of cell types have found that $PM_{2.5}$ promoted a proinflammatory responses though AhR[74, 75]. In addition to affecting AhR and inflammatory pathways, $PM_{2.5}$ exposure also causes epigenetic changes. Specifically, $PM_{2.5}$ exposure has been shown to alter levels of microRNA[76], expression of long non-coding RNA[77], and patterns of DNA methylation[78, 79, 80, 81, 82]. Finally, rather than working through any particular pathway or receptor, $PM_{2.5}$ can damage DNA directly. DNA adduct formation, apoptosis, dysfunctional DNA repair mechanisms, and induction of double-strand breaks have all been observed following $PM_{2.5}$ exposure[83, 84].

1.8 Dissertation Aims

This study attempts to determine the effect that $PM_{2.5}$ exposure has on fibroblast biology, with a particular focus on its effect on fibroblast activation and myofibroblast differentiation. Our central hypothesis is that $PM_{2.5}$, at low concentrations and with repeated exposure, potently induces fibroblasts to undergo myofibroblast differentiation, and this ultimately plays an important role in the development and susceptibility to lung fibrosis. Furthermore, we hypothesize this occurs through an NF-κB and BMP2-dependent mechanism. We will test these hypotheses through 4 Specific Aims:

Specific Aim 1: To delineate the conditions and determinants by which $PM_{2.5}$ affects fibroblast-to-myofibroblast differentiation and determine the upstream signaling pathways by which it does so. Although some studies suggest that $PM_{2.5}$ leads to fibroblast activation, how it does so is unknown and the conditions by which it does so vary among studies. Here, we sought to elucidate the specific conditions by which $PM_{2.5}$ leads to myofibroblast differentiation. Furthermore, $PM_{2.5}$ has been shown to signal through a variety of signaling pathways. Here, we

hypothesize that $PM_{2.5}$ exposure will promote an increase in myofibroblast differentiation, specifically through NF-κB activation.

Specific Aim 2: To determine the effect of $PM_{2.5}$ exposure on BMP2 expression and secretion, and to determine the role of BMP2 on $PM_{2.5}$-induced myofibroblast differentiation. Prior research has shown that $PM_{2.5}$ increases BMP2 in other cell types and that BMP2 is important in cellular differentiation and organogenesis, though the effects on fibroblast are unclear. Here, we hypothesize that exposure to $PM_{2.5}$ will increase BMP2 and that this plays a major role in promoting myofibroblast differentiation.

Specific Aim 3: To perform a broad analysis of the cytokine secretion and DNA methylation changes $PM_{2.5}$ causes in lung fibroblasts. In the first two Aims, we focus on a specific fibroblast phenotype (i.e. myofibroblast differentiation) that might be affected by $PM_{2.5}$ exposure, and a specific cytokine (BMP2) that might be responsible for this. However, we recognize that $PM_{2.5}$ might induce other, long-lasting epigenetic changes in these cells and cause the release of other cytokines, in addition to BMP2. In this Specific Aim, we specifically analyze cells on a DNA methylation array and supernatants on a Luminex cytokine kit to assess other cytokines and epigenetic changes that might occur in fibroblasts exposed to $PM_{2.5}$. The results collected in this Aim will provide additional context for our previous data and assist with generating new hypotheses for future investigation

Specific Aim 4: To determine if $PM_{2.5}$ exposure *in vivo* increases susceptibility to pulmonary fibrosis. Our prior *in vitro* experiments suggest that $PM_{2.5}$ exposure promotes myofibroblast differentiation, an important process in fibrogenesis. We hypothesis that these *in vitro* results will translate *in vivo*, and that mice exposed to $PM_{2.5}$ will develop worse fibrosis through its effects on fibroblast activation.

1.9 Tables

Table 1.1 Effects of PM on fibroblast biology

Citation	Cell Line	Dose	Duration	Result
46	HFL-1	100-200 μg/ml	24 hours	Increases in αSMA, Col1, and TGF-β1
63	Human embryo lung fibroblasts	100 μg/ml	24 hours	Increases in mitochondrial superoxide; Decreases in Intracellular ATP
64	HEL 299	50 μg/ml	72 hours	Increase in mitochondrial disfunction and metabolic disruption
65	HEL 12469	1 μg/ml*, 20 μg/ml*	24 hours	Increase NF-κB activation
66	Primary nasal fibroblasts	200 μg/ml, 200 μg/ml	1 hour	Increase in ROS production
67	Primary nasal fibroblasts	25 μg/ml, 100 μg/ml, 200 μg/ml	24 hours	Increase in proinflammatory cytokines
62	MRC-5 human lung fibroblast cells	62.5 μg/mL**	24, 48, and 72 hours	Increase in autophagy as measured by expression of autophagy related genes and expression of pro-caspase 3
61	Human pulmonary fibroblasts (HPF-a)	50 μg/cm^2**	Various timepoints between 6 and 72 hours	Increase in endoplasmic reticulum stress through activation of the sigma-1 receptor

*Extractable Organic Matter from $PM_{2.5}$, **Silica particles

Chapter 2 Repeated, Low Concentration Exposure to $PM_{2.5}$ Promotes Myofibroblast Differentiation through NF-κB

2.1 Abstract

Air pollution accounts for 6.67 million premature deaths worldwide each year. Over half of these deaths are attributable to ambient $PM_{2.5}$ exposure; with mortality projected to further increase in the coming decades. Although the effect of $PM_{2.5}$ exposure on the incidence of many diseases, particularly lung diseases, is well understood, the specific mechanisms at play are often less clear. Fibroblasts are the most common mesenchymal cell type in the lung and are involved in maintaining lung homeostasis and directing repair. The effects of $PM_{2.5}$ exposure, although extensively studied in airway epithelium, are not as well studied in fibroblasts. We sought to elucidate the effect of $PM_{2.5}$ exposure on pulmonary fibroblasts. We treated lung fibroblasts *in vitro* with $PM_{2.5}$ at varying concentrations for varying lengths of time, and measured markers of myofibroblast differentiation, such as α-smooth muscle actin (SMA) and collagen I (Col1a1). We observed that treatment of lung fibroblasts with $PM_{2.5}$ resulted in increased expression of αSMA and Col1a1. Notably, this increase occurred in response to repeated, but not single $PM_{2.5}$ exposures and at low $PM_{2.5}$ concentrations, often lower than that used in other studies. $PM_{2.5}$ triggers a variety of signal pathways, including activation of AhR and NF-κB. Treatment of fibroblasts with the NF-κB inhibitor IMD 0354 demonstrated that induction of myofibroblast differentiation by $PM_{2.5}$ can be completely abolished by inhibition of NF-κB. These data demonstrate how low concentrations of repeated $PM_{2.5}$ exposures, can have potent effects on

myofibroblast differentiation and play potentially important roles in the development of pulmonary fibrosis.

2.2 Introduction

Air pollution contributes to approximately 6.67 million premature deaths per year worldwide[1]. This number is expected to double by 2050 if no further attempts to curb current levels of pollution are made[85]. Over half of the deaths (4.14 million) attributable to air pollution are specifically due to $PM_{2.5}$[1, 4]. Many of these deaths are attributable to lung diseases, such as COPD, lung cancer, asthma, and fibrosis. As such, there is an extensive literature examining the effects that $PM_{2.5}$ exposure has on different cell types in the lung. Many of these studies focused on epithelial cells, as they are the first line of defense against environmental insults. These studies have found that $PM_{2.5}$ exposure increases ROS levels, induces the release of proinflammatory cytokines such as IL-6, IL-8, and CCL2, activate TGFβ, increase cell contractility, and promote EMT[35, 43, 44, 45, 46].

The effect that $PM_{2.5}$ exposure has on other cells within the lung, such as fibroblasts and other mesenchymal cells, is not as extensively described. That said, prior work has shown that mitochondrial dysfunction[63, 64], disruptions in cellular metabolism[64], NF-κB activation[65], ROS production[66], and secretion of proinflammatory cytokines[67] are all increased following $PM_{2.5}$ exposure in fibroblasts. Additionally, one study found that fibroblasts exposed directly to $PM_{2.5}$ increased activation of the TGF-β1/SMAD3 pathway, secreted more TGF-β1, and differentiated into myofibroblasts, as measured by increases in αSMA and Col1a1[46]. While this was the first study to show that direct $PM_{2.5}$ exposure could promote myofibroblast differentiation, it was limited in that the investigators looked at single timepoint following a single exposure. Further

work that characterizes the effect that $PM_{2.5}$ exposure has on myofibroblast differentiation is necessary.

$PM_{2.5}$ operates through a diverse array of mechanisms and mediators. One of the main ways that $PM_{2.5}$ interacts with cells is by increasing ROS and oxidative stress, which in turn activates NF-κB. This has been shown in a variety of contexts, including *in vitro* experiments involving vocal fold fibroblasts[86], alveolar and bronchial epithelial cells[87, 88], and macrophages[87], as well as mouse studies where $PM_{2.5}$ exposure led to NF-κB activation *in* vivo[87, 89]. $PM_{2.5}$ contains a variety of xenobiotic chemicals, including aromatic hydrocarbons, that are known to activate AhR. $PM_{2.5}$ modulates a variety of cellular processes through AhR signaling including the production and release of ROS and proinflammatory cytokines in both fibroblasts and epithelial cells[74, 75]. The signaling pathways that are responsible for $PM_{2.5}$ induced activation of fibroblasts and myofibroblast differentiation are unknown.

We sought to investigate how PM exposure affects the differentiation of fibroblasts to myofibroblasts and the signaling mechanisms by which it does so. We hypothesize that the exposure of fibroblasts to $PM_{2.5}$ causes fibroblasts to differentiate into myofibroblasts, a process which contributes to the development of pulmonary fibrosis. Furthermore, we believe that this process will be dependent on NF-κB signaling.

2.3 Methods

Cell Culture

Normal primary human lung fibroblasts (CCL210, American Type Culture Collection, Manassas, VA) were cultured in DMEM (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% FBS and 1% penicillin/streptomycin and maintained in a 37° C incubator with 5% CO_2.

$PM_{2.5}$ Collection and Preparation

We treated fibroblasts with two sources of $PM_{2.5}$. $PM_{2.5}$ from the National Institute of Standards and Technology (NIST, SRM 1649b, Gaithersburg, MD) – referred to as NIST-$PM_{2.5}$ – was collected from 1976 in Washington, D.C. and is a well-characterized source of $PM_{2.5}$ that has been used by many investigators over the years[90]. NIST-$PM_{2.5}$ was solubilized in double distilled water and sonicated (VWR, model no. 97043–968, VWR International, Radnor, PA, USA) for at least 75 minutes prior to use.

$PM_{2.5}$ was also collected from a manual sampler located on the rooftop of the Peking University School of Public Health in Beijing (Beijing-$PM_{2.5}$); this location was chosen because of its high levels of traffic, diesel, and urban-related pollution. Beijing-$PM_{2.5}$ was collected on 90 mm Emfab filters, consisting of borosilicate fibers reinforced with woven cloth and bonded with polytetrafluoroethylene (TX40HI20WW, part #7234, Pall Company, Beijing Office, Beijing, China). These filters were replaced every 24 hours. After collection, the filters were folded in half and shipped to the US in sterile secure packaging and stored at −20° C until extraction.

Beijing-$PM_{2.5}$ was extracted from the filters as previously described[91]. Prior to extraction, each filter was equilibrated in sterile amber jars located in a sterile biosafety containment hood at constant humidity and room temperature for 24 hours. Each filter was weighed on a microbalance (AC 100, Mettler-Toledo, Columbus, OH) before extraction. To extract $PM_{2.5}$, each filter was placed face down in amber jars, wetted with 20 ml of double distilled water, and sonicated on ice at 15 min intervals for a total of 3 hours. After sonication, filters were air-dried in amber jars located in the same biosafety containment hood at constant humidity and room temperature for 3 days before being weighed on a microbalance. The difference in weight

(averaged from 3 to 5 measurements) before and after extraction was used to calculate the concentration (mg/ml). Samples were aliquoted and stored for future use at −80° C.

Treatment of cells with $PM_{2.5}$

Prior to treatment, the cells were plated at 2.5×10^5 cells/well in 6-well plates overnight. Medium was subsequently removed, and the cells were incubated for approximately 24 hours in serum-free DMEM. Cells were then treated with various concentrations of $PM_{2.5}$ (0.01 – 50 $\mu g/cm^2$), diluted in DMEM, for the indicated times. For certain experiments, cells were treated with repeated doses of $PM_{2.5}$. In these instances, medium was removed before each dose, cells were washed with PBS, and fresh medium with $PM_{2.5}$ (0.01 – 10 $\mu g/cm^2$) was added. Control cells in these repeated exposure experiments had medium replaced with DMEM alone. After the exposure period(s), cells were harvested for RNA or protein analysis. In some experiments, the cells were pre-incubated with the aryl hydrocarbon receptor (AhR) antagonist CH223191 (10 μM, Tocris Bioscience, Ellisville, MO) or the NF-κB inhibitor IMD 0354 (10nM, Tocris Bioscience, Bristol, UK) for 30 min prior to addition of $PM_{2.5}$. The concentration of the inhibitors used to treat cells were chosen based on literature[92, 93, 94].

Cytotoxicity

Cell cytotoxicity, as measured by levels of lactate dehydrogenase (LDH) in the supernatant, was assessed using the LDH Cytotoxicity Assay Kit (Catalog number 88953, Thermo Fisher Scientific, Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions.

RNA Extraction and quantitative real-time PCR

Trizol (Catalog Number 15596018, Invitrogen, Carlsbad, CA, USA) was used to isolate RNA according to manufacturer's instructions. Total RNA was quantified with a Nanodrop

Spectrophotometer (NanoDrop 2000, Nanodrop Technologies LLC, Thermo Fisher Scientific, Wilmington, DE, USA). Isolated RNA was stored at −80° C until processing. RNA was reverse-transcribed by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's recommendations. Quantitative real-time PCR was performed on the resulting cDNA using SYBR green PCR Master Mix (Applied Biosystems) on a StepOne Real-time PCR System (Applied Biosystems). Primer specificity was verified by observing a single peak on the melting curve. The fold change in expression of the target genes was calculated by the ΔΔCt method relative to β-actin as the endogenous control. Table 2.1 lists the primers used.

Immunoblot Analysis

Fibroblasts were lysed in lysis buffer (PBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN) and Phosphatase Inhibitor I and II Cocktails (EMD Millipore, Billerica, MA). Protein concentration was quantified with the DC Protein Assay (5000111, Biorad, Hercules, CA) and equal protein was loaded onto either 8% or 10% tris-glycine gels, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were blocked with 5% bovine serum albumin before being probed overnight at 4° C with the antibodies listed in Table 2.2. Membranes were then incubated with appropriate secondary antibody conjugated to horseradish peroxidase (Cell Signaling) for 1 hour at room temperature before developing with enhanced chemiluminescent reagent (GE Healthcare, Pittsburgh, PA). For all protein bands, densitometry was analyzed by Image J Software (NIH, Bethesda, MD) and normalized to α-tubulin or GAPDH.

Statistical Analysis

The statistical significance of the quantitative real-time PCR data was determined by performing an analysis of variance (ANOVA) in Prism (GraphPad Prism Software v7, La Jolla, CA). Western Blot data was quantified by densiometric methods and significance tested via ANOVA. Dunnett's test was used for post hoc testing in both instances.

2.4 Results

Single exposure to $PM_{2.5}$ exposure did not result in any increase in myofibroblast differentiation

Previous studies have shown that a single exposure of $PM_{2.5}$ induces myofibroblast differentiation in a concentration-dependent manner[46]. In attempt to replicate these findings, we exposed fibroblasts to increasing concentrations of $PM_{2.5}$ (0.1 – 30 μg/cm^2) and collected protein to assess expression of αSMA, a marker of myofibroblast differentiation (Figure 2.1A). Interestingly, neither Beijing-$PM_{2.5}$ nor NIST-$PM_{2.5}$ induced any increase in αSMA levels after 24, 48, or 72 hours of treatment (72-hour data shown in Figure 2.1). These results were surprising as prior work[46] found that dose dependent increases in αSMA occurred following a single $PM_{2.5}$ exposure. To ensure that $PM_{2.5}$ did not induce cytotoxicity, we measured levels of lactate dehydrogenase (LDH) activity in the supernatant. No changes in LDH activity were observed in any of the $PM_{2.5}$ exposed cells (Figure 2.2).

Repeated $PM_{2.5}$ exposures promoted an increase in myofibroblast differentiation

Although we did not observe an increase in αSMA after a single $PM_{2.5}$ exposure, important biologic changes, such as mitochondrial disruption and increased inflammatory response have often been demonstrated in both *in vivo* and *in vitro* models utilizing repeated dosing[95, 96]. Real-world exposures among humans also typically involve daily exposure to $PM_{2.5}$ over long periods. We thus sought to determine how fibroblasts would be affected by repeated

exposures to multiple concentrations of $PM_{2.5}$ over several days. We first treated fibroblasts with repeated doses of $PM_{2.5}$ every day for six days (Figure 2.3A). Lower doses of $PM_{2.5}$ were specifically chosen to more closely mimic the daily exposures encountered in the real world and limit the potential *in vitro* toxicity that could occur from repeated exposure. To limit the effects of additive accumulation of $PM_{2.5}$ with repeat dosing, cells were washed with PBS before every exposure. In contrast to a single exposure, repeated exposure to NIST-$PM_{2.5}$, even at much lower doses, induced an increase in αSMA (Figure 2.3B). To determine the minimal number of exposures necessary to induce myofibroblast differentiation, fibroblasts were treated with just two doses of $PM_{2.5}$, separated 72 hours apart (Figure 2.3C). Both Beijing-$PM_{2.5}$ (Figure 2.3D/2.3E) and NIST-$PM_{2.5}$ (Figure 2.3F/2.3G) induced significant, dose dependent increases in both αSMA and collagen. By comparison, no significant increase in αSMA was observed after single exposures to Beijing-$PM_{2.5}$ (Figure 2.3I) or NIST-$PM_{2.5}$ (Figure 2.3J) when taken out to 6 days. Taken together, these data show that repeated, but not single, $PM_{2.5}$ exposures induces myofibroblast differentiation. Notably, while both Beijing-$PM_{2.5}$ and NIST-$PM_{2.5}$ increased myofibroblast differentiation, NIST-$PM_{2.5}$ appeared more potent, inducing increases in αSMA and collagen at a lower concentration compared to Beijing-$PM_{2.5}$.

The increase in αSMA protein was preceded by an increase in mRNA

To determine whether the increase in αSMA and collagen I after repeated exposure to $PM_{2.5}$ was preceded by an increase in mRNA levels, levels of ACTA2 and COL1A1 transcripts were assayed by RT-PCR after the first and second exposure to NIST-$PM_{2.5}$ (Figure 2.4A). A single $PM_{2.5}$ exposure did not induce an increase in *ACTA2* or *COL1A1* transcript (data not shown), but levels of *ACTA2* mRNA increased after a second $PM_{2.5}$ exposure. Interestingly, *COL1A1* transcript levels did not increase following repeated NIST-$PM_{2.5}$ exposure (Figure

2.4B/2.4C). These data indicate that while $PM_{2.5}$ stimulates αSMA through increases in *ACTA2* transcript levels, the increase in Col1a1 by $PM_{2.5}$ occurs post-transcriptionally.

The $PM_{2.5}$ induced increase in myofibroblast differentiation is dependent on NF-κB signaling

$PM_{2.5}$ has been shown to activate a variety of signaling pathways, including the aryl hydrocarbon receptor (AhR) and nuclear factor (NF)-κB signaling[74, 75, 87, 88]. To determine if NF-κB is activated after low concentration $PM_{2.5}$ exposure, we examined NF-κB nuclear localization following repeated NIST-$PM_{2.5}$ exposure. Following repeated NIST-$PM_{2.5}$ exposure, the p65 subunit of NF-κB shifted from the cytoplasm to the nucleus (Figure 2.5A/2.5B), indicating that NF-κB is indeed activated following repeated NIST-$PM_{2.5}$ exposure.

To determine which pathway – AhR vs NF-κB – is responsible for the ability of $PM_{2.5}$ to promote myofibroblast differentiation, we pretreated fibroblasts with CH223191, which inhibits AhR, or IMD 0354, which inhibits NF-κB, prior to treatment with repeated doses of NIST-$PM_{2.5}$ (Figure 2.6A & 2.5C, respectively). The AhR inhibitor itself raised basal levels of αSMA and was unable to inhibit the ability of NIST-$PM_{2.5}$ to stimulate αSMA expression (Figure 2.6B). By contrast, the NF-κB inhibitor blocked the NIST-$PM_{2.5}$-induced increase in both αSMA and Col1a1 (Figure 2.5D – 2.5G), indicating that the ability of $PM_{2.5}$ to induce myofibroblast differentiation is dependent on NF-κB signaling.

2.5 Discussion

Here, we show that $PM_{2.5}$ activates fibroblasts and induces myofibroblast differentiation. We found that particulate exposure increases expression of αSMA and Col1a1, markers of myofibroblast differentiation. Notably, this increase occurred in response to repeated but not single $PM_{2.5}$ exposures. Additionally, these effects occurred at relatively low concentrations of

$PM_{2.5}$, which supports recent findings suggesting that lower concentrations of $PM_{2.5}$ than previously realized can have significant bioactive effects[24, 25, 26, 27]. We also sought to determine the signaling mechanisms by which $PM_{2.5}$ induces myofibroblast differentiation. PM is recognized to trigger a variety of signaling pathways, including the activation of AhR and NF-κB[73, 97, 98]. Our data demonstrated that the $PM_{2.5}$-induced increase in myofibroblast differentiation was dependent on NF-κB activation and not via activation of AhR.

We observed that $PM_{2.5}$ increased expression of αSMA and collagen only after repeated exposure to $PM_{2.5}$. We believe this was not simply a result of the quantitative sum of $PM_{2.5}$ as cells were treated with much lower concentrations of $PM_{2.5}$ than experiments utilizing a single exposure, and cells were washed between $PM_{2.5}$ exposures. In addition, we found that just two exposures to $PM_{2.5}$ was sufficient to cause an increase in αSMA and collagen to levels similar to that found with repeated exposure every day for six days. These data suggest that cells may undergo priming after the first exposure that is necessary for them to differentiate into myofibroblasts upon further environmental insult.

We observed that two independent sources of $PM_{2.5}$, Beijing-$PM_{2.5}$ and NIST-$PM_{2.5}$, were both capable of inducing expression of αSMA and increasing collagen I in fibroblasts, though the effective concentrations that produced similar effects were at different orders of magnitude (1.0 μg/cm^2 or 4.75 μg/ml for Beijing-$PM_{2.5}$ and 0.01-0.1 μg/cm^2 or 0.0475-0.475 μg/ml for NIST-$PM_{2.5}$). Nonetheless, these concentrations were both significantly lower than that used in other studies[46]. Interestingly, our data are also in contrast with other studies that have previously shown that $PM_{2.5}$ activates the TGF-β1/SMAD3 pathway, increases TGF-β1 production, and differentiates fibroblasts to myofibroblasts after a single exposure to high concentrations of $PM_{2.5}$[46], which we were not able to observe at any of the higher concentrations

utilized. The reason for the difference in our results, may be due to differences in the source of $PM_{2.5}$ used. This may cause different signaling pathways to be responsible for driving myofibroblast differentiation in a single exposure to high concentrations of $PM_{2.5}$ compared to repetitive exposure to lower $PM_{2.5}$ concentrations. Future research can attempt to address this question by developing effective $PM_{2.5}$ exposure protocols that cause myofibroblast differentiation for both "high dose, single exposure" and "low dose, repeat exposure" methods and then directly comparing the underlying mechanisms driving myofibroblast differentiation in both methods.

Activation of NF-κB in fibroblasts has been shown to be capable and sufficient to induce myofibroblast. Treatment of fibroblasts with an NF-κB inhibitor or knockdown of the p65 subunit of NF-κB suppressed TGF-β1-induced myofibroblast differentiation[99]. Multi-walled carbon nanotubes were shown to simultaneously activate NF-κB signaling and increase the expression of the profibrotic mediators TIMP1 and OPN in mouse lung fibroblasts, suggesting a link between NF-κB activation and fibrosis *in vivo*[100]. Two independent studies found that NF-κB activation promotes an apoptosis resistant phenotype in myofibroblasts[101, 102]. Blocking NF-κB signaling in bleomycin exposed mice with a pharmacological inhibitor resulted in less severe fibrosis[103]. Won *et al*. showed that exposure to urban PM caused increases in myofibroblast differentiation in vocal cord fibroblasts. Specifically, PM exposure was shown to activate the MAPK/NF-κB signaling pathway through ROS generation[86]. Our study adds to this growing body of literature by showing that activation of NF-κB following $PM_{2.5}$ exposure is necessary for $PM_{2.5}$ to promote myofibroblast differentiation.

The underlying reason that repeated $PM_{2.5}$ exposures is necessary for induction of myofibroblast differentiation remains unclear. Our NF-κB activation experiments show that NF-

κB is activated by repeated $PM_{2.5}$ exposure. It is possible that the initial activation occurs through another, independent mechanism following the first exposure. This causes changes in the fibroblasts that make them more susceptible to myofibroblast differentiation following the second exposure. These other mechanisms may include activation of other inflammatory pathways like MAPK or NLRP3[104], damage to the cell itself, such as mitochondrial damage, lipid oxidation, or DNA damage[105, 106], the release of growth factors like TGF-β1 or BMP2[46, 107], or epigenetic changes[81, 91]. Future studies examining the changes that occur following the initial NF-κB activation would be a logical first step in determining why multiple exposures are needed to cause myofibroblast differentiation.

Future studies could investigate further by utilizing a similar exposure protocol and the appropriate modifications to experiment design to continue to probe deeper mechanistically. For example, follow-up studies could inhibit NF-κB signaling prior to either the first or second exposure specifically, in our 6 day repeated exposure protocol. Differences in the effect of inhibiting the first $PM_{2.5}$ exposure versus inhibiting the second would allow us to make further inferences about the role and timing of NF- κB activation in the increase in myofibroblast differentiation following repeated $PM_{2.5}$ exposure.

Our study demonstrated that very low concentrations of $PM_{2.5}$ were sufficient to induce αSMA expression, but how this translates to real-world exposure is not clear. As fibroblasts sit on the basolateral side of alveoli and are protected by alveolar epithelial cells, mathematical estimates of *in vitro* concentrations based on *in vivo* exposure[58] as often used in other studies are more are difficult to predict in fibroblasts. Nonetheless, we do believe lung fibroblasts can be directly exposed to $PM_{2.5}$ during real-world exposures as $PM_{2.5}$ has been demonstrated to break down alveolar barrier function[59, 60] and cause systemic disease. This is further supported by

increasing epidemiologic data demonstrating that low concentrations of $PM_{2.5}$ below limits set forth by many countries are sufficient to cause deleterious health outcomes and induce systemic diseases such as cardiovascular disease and cancer[24, 26, 27, 69].

As fibroblasts are critical to repair and regeneration, the finding that $PM_{2.5}$ directly results in myofibroblast differentiation could serve as a potential mechanism for how $PM_{2.5}$ might contribute to the development or progression of pulmonary fibrosis. Previous studies have shown that $PM_{2.5}$ exposure can promote fibrosis in a mouse model[46, 88, 165]. Future *in vivo* studies should seek to build on this by elucidating the mechanisms driving fibrosis in these mouse models. Epidemiologic studies have shown that exposure to PM is associated with the development and progression of idiopathic pulmonary fibrosis (IPF). PM has been shown to contribute to acute exacerbations of IPF[48, 49], enhanced mortality due to IPF[49], and accelerated lung function decline in IPF patients[50]. Our *in vitro* results suggest that the mechanism may involve NF-κB activation in the fibroblasts prior to myofibroblast differentiation.

In conclusion, we show that repeated exposure to low concentrations of $PM_{2.5}$ from multiple sources promoted myofibroblast differentiation through an NF-κB dependent mechanism. These results contribute to an already large and ever-growing body of literature showing the negative health effects of $PM_{2.5}$ in general[4, 11, 12, 13, 18, 19, 22]. These results also provide important insights into fibroblast biology by showing the necessary exposure conditions and underlying mechanism through which $PM_{2.5}$ can affect fibrogenesis and myofibroblast differentiation.

2.6 Tables

Table 2.1: Primers utilized in Aim 1

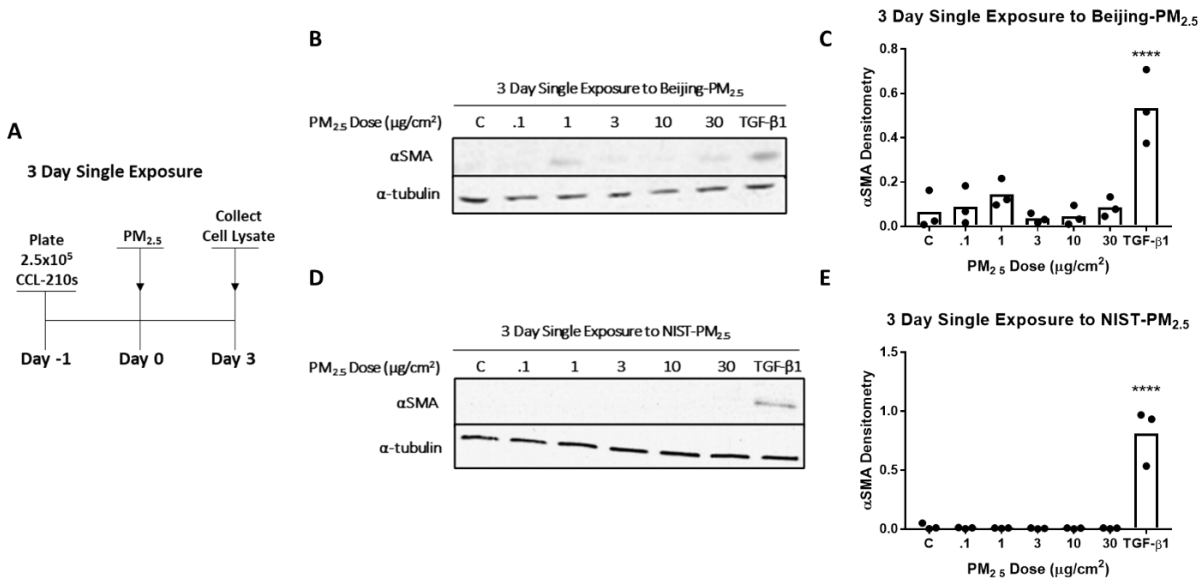
Gene	Primer Sequence
β-actin	Forward TAGTTGCGTTACACCCTTTC Reverse GCACGAAGGCTCATCATT
ACTA2	Primers with specific probes used to amplify and detect ACTA2 (Hs00426835_g1) were obtained from Applied Biosystems
COL1A1	Forward GGGCAAGACAGTGATTGAAT Reverse GGAGTTTACAGGAAGCAGACA

Table 2.2: Antibodies utilized in Aim 1

Protein	Dilution	Company
GAPDH	1:1000	Santa Cruz, Dallas, TX, USA
α-tubulin	1:4000	Sigma-Aldrich, St. Louis, MO, USA
P65	1:1000	Cell Signaling, Danvers, MA, USA
Lamin B	1:1000	Cell Signaling, Danvers, MA, USA
Col1a1	1:1000	Invitrogen, Waltham, MA, USA
αSMA	1:1000	Dako Brand, Agilent Technologies, Santa Clara, CA, USA

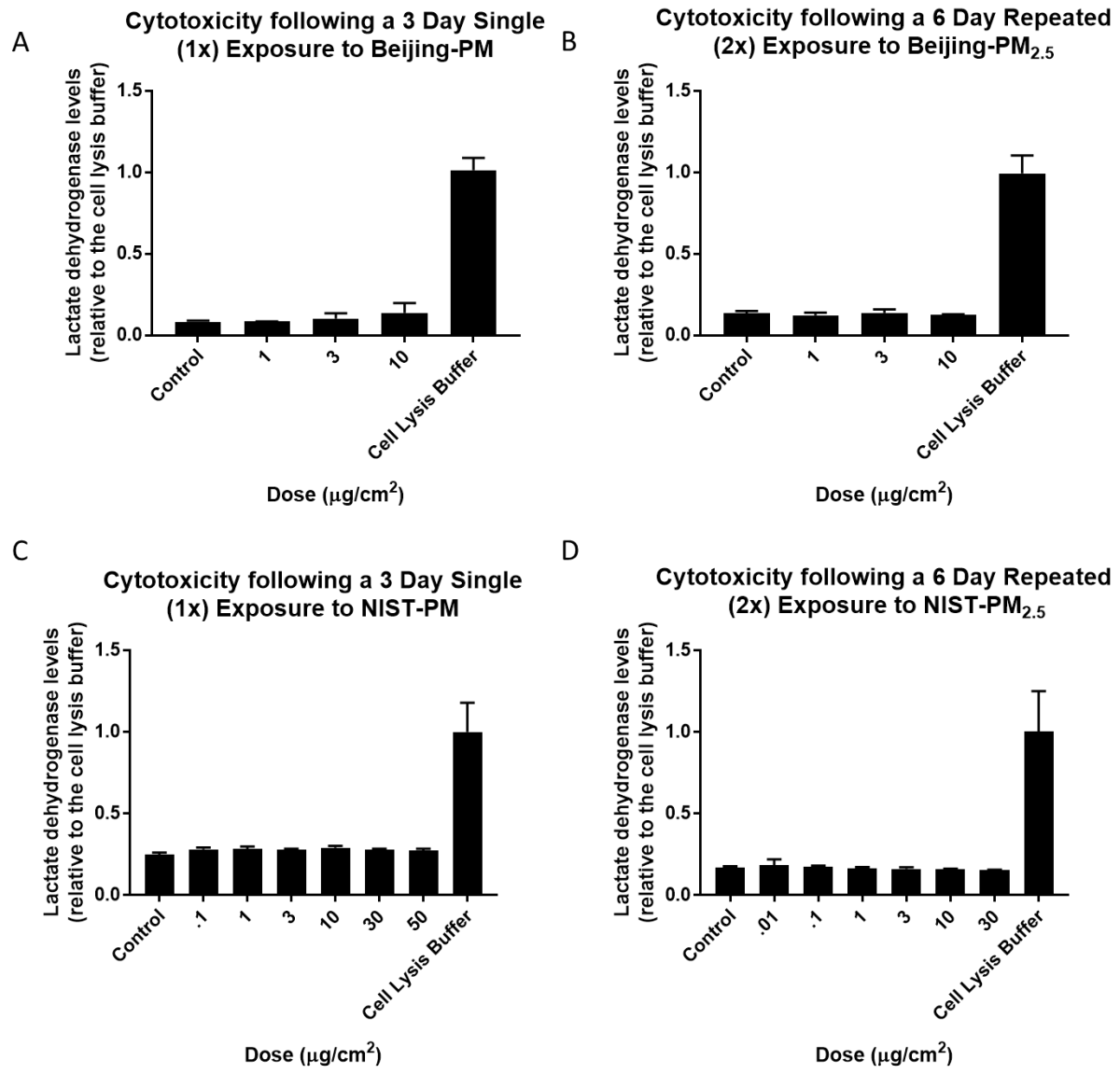
2.7 Figures

Figure 2.1: Single exposure to $PM_{2.5}$ did not result in any increase in myofibroblast differentiation



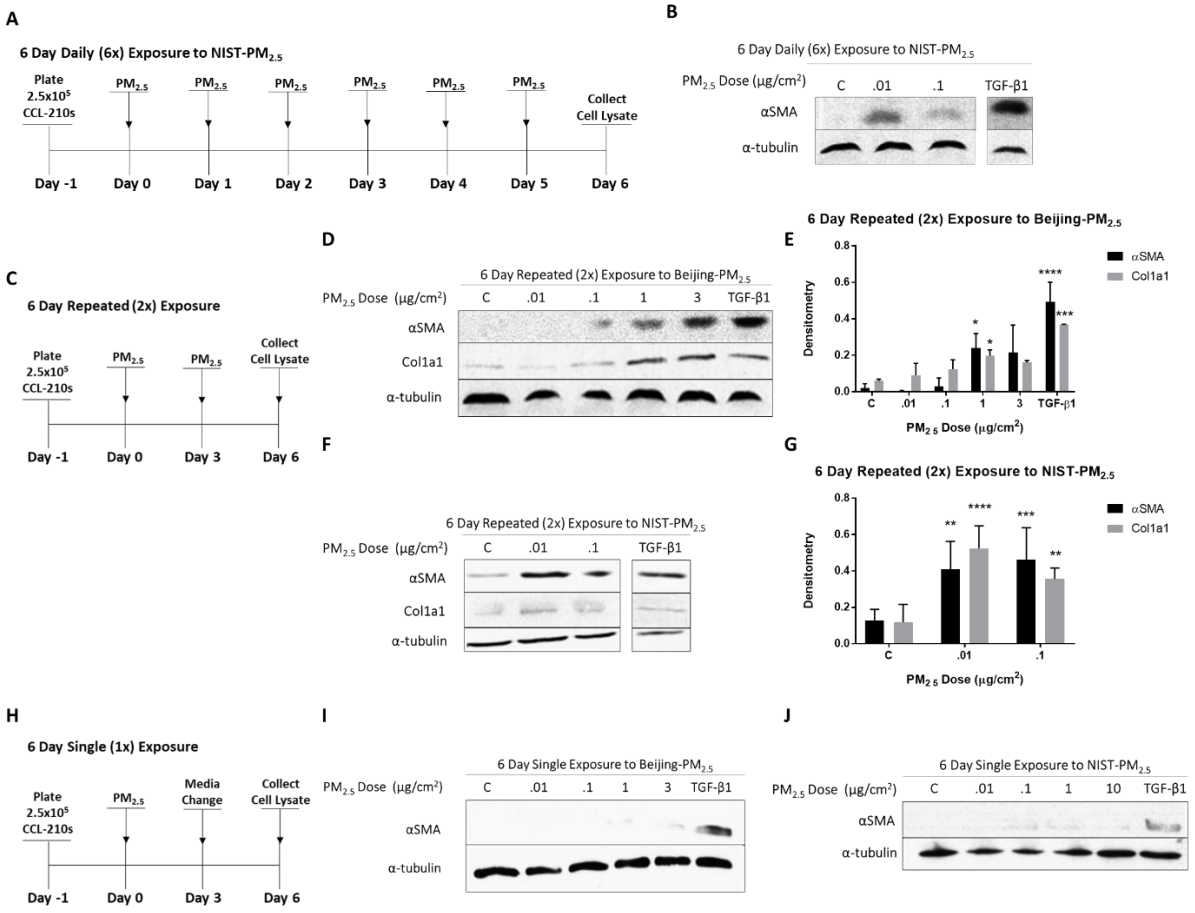
Legend - αSMA protein levels following a 3 day single exposure to Beijing or NIST $PM_{2.5}$. A) A wire diagram depicting the 3 day single exposure protocol in which CCL-210 fibroblasts were treated with various concentrations of Beijing-$PM_{2.5}$ or NIST-$PM_{2.5}$. B) and D) Representative immunoblots for αSMA protein expression following 3 day single exposure to Beijing and NIST $PM_{2.5}$, respectively. C) and E) Densitometry for αSMA protein expression following 3 day single exposure to Beijing and NIST $PM_{2.5}$, respectively. ****$p < 0.00005$ relative to control.

Figure 2.2: $PM_{2.5}$ exposure protocols did not cause an increase in lactate dehydrogenase



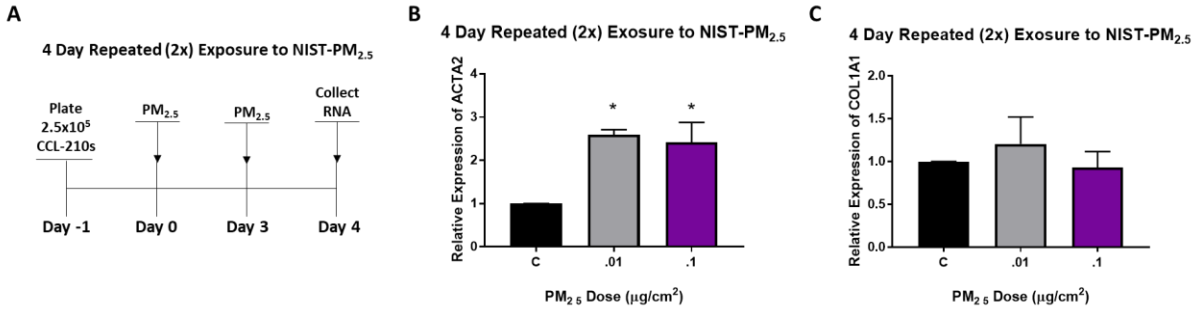
Legend – Lactate dehydrogenase levels following a variety of $PM_{2.5}$ exposures, specifically, the A) 3 day single exposure to Beijing-$PM_{2.5}$, B) 6 day repeated exposure to Beijing-$PM_{2.5}$, C) 3 day single exposure to NIST-$PM_{2.5}$ and D) 6 day repeated exposure to NIST-$PM_{2.5}$

Figure 2.3: Repeated $PM_{2.5}$ exposures promoted an increase in myofibroblast differentiation



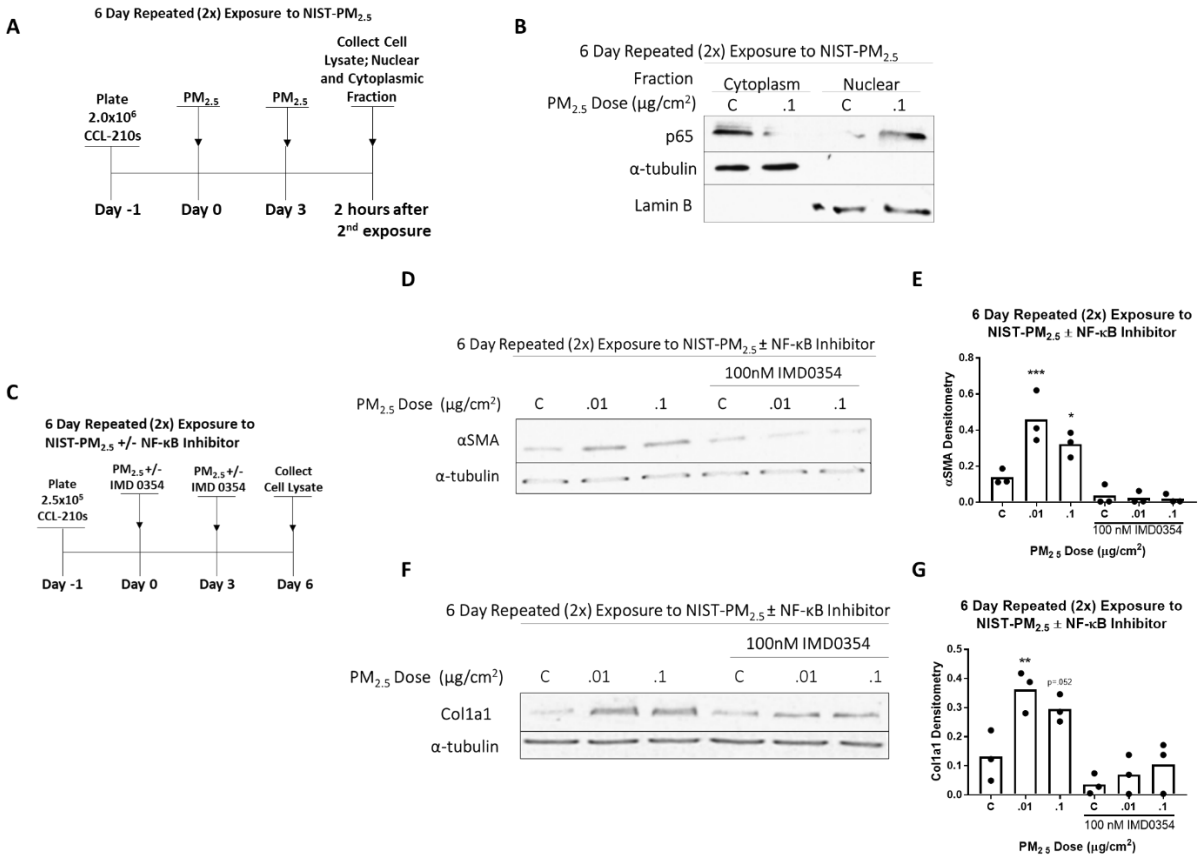
Legend – αSMA and Collagen protein levels following 6 day repeated exposures to Beijing and NIST $PM_{2.5}$. A), C), and H) Wire diagrams depicting the 6 day daily, repeated, and single exposure protocols, respectively. B) Representative immunoblot showing αSMA protein expression following 6 day daily exposure to NIST-$PM_{2.5}$. D) and F) Representative immunoblots of αSMA and Collagen protein levels following 6 day repeated exposure to Beijing or NIST $PM_{2.5}$, respectively. E) and G) Densitometry of αSMA and Collagen protein levels following 6 day repeated exposure to Beijing or NIST $PM_{2.5}$, respectively. I) and J) Representative immunoblots showing αSMA protein levels following 6 day single exposure to Beijing or NIST $PM_{2.5}$, respectively. *$p<0.05$, **$p<0.005$, ***$p<0.0005$, ****$p < 0.00005$ relative to control.

Figure 2.4: The increase in αSMA protein was preceded by an increase in mRNA



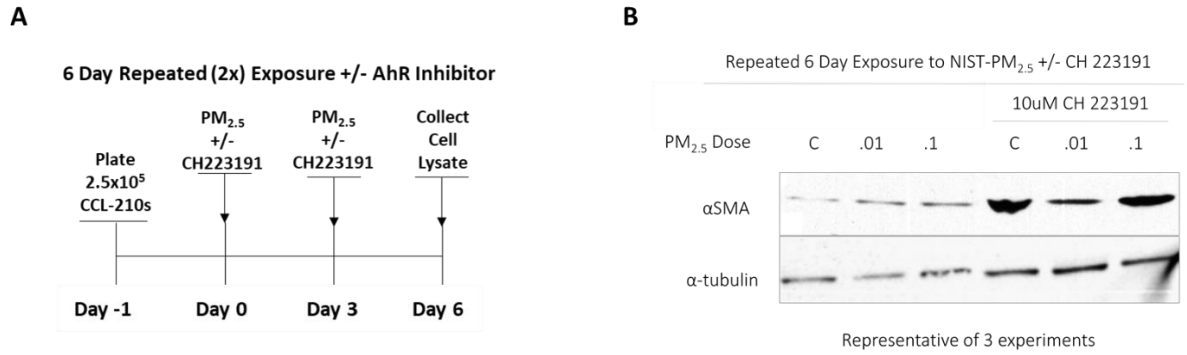
Legend – ACTA2 and COL1A1 mRNA levels following 4 day, repeated $PM_{2.5}$ exposure. A) A wire diagram depicting the 4 day single exposure protocol in which CCL-210 fibroblasts were exposed to NIST-$PM_{2.5}$. B) Expression of ACTA2 transcript following repeated $PM_{2.5}$ exposure as measured by rt-qPCR. C) Expression of COL1A1 transcript following repeated $PM_{2.5}$ exposure as measured by rt-qPCR (housekeeping gene – β-actin). *p<.05 relative to control.

Figure 2.5: The $PM_{2.5}$ induced increase in myofibroblast differentiation is dependent on NF-κB signaling



Legend – The impact of NF-κB on repeated $PM_{2.5}$ exposure. A) and C) Wire diagrams depicting the alterations to the 6 day repeated exposure protocol made in this experiment. B) Representative immunoblot showing the localization of the p65 subunit of NF-κB following 6 day repeated low concentration NIST-$PM_{2.5}$ exposure. D) and F) Representative immunoblots showing αSMA and Collagen protein levels, respectively, following 6 day repeated exposure to NIST-$PM_{2.5}$ +/- IMD 0354. E) and G) Densitometry showing αSMA and Collagen protein levels, respectively, following 6 day repeated exposure to NIST $PM_{2.5}$ +/- IMD 0354. *$p<0.05$, **$p<0.005$, ***$p<0.0005$ relative to control.

Figure 2.6 The $PM_{2.5}$-induced increase in myofibroblast differentiation is not dependent on AhR signaling



Legend – The effect of CH223191 on the $PM_{2.5}$-induced increase in myofibroblast differentiation. A) Wire diagram of the 6 day repeated exposure to NIST $PM_{2.5}$ +/- CH223191. B) Representative immunoblots showing αSMA protein levels following 6 day repeated exposure to NIST $PM_{2.5}$ +/- CH223191.

Chapter 3 Bone Morphogenic Protein 2, a Secreted Cytokine Upregulated by $PM_{2.5}$ Exposure, Modulates Fibroblast Biology and Myofibroblast Differentiation

3.1 Abstract

Here, we sought to build off the work in our previous Aim, where we demonstrated that repeated exposure to low concentrations of $PM_{2.5}$ increased myofibroblast differentiation through NF-κB. NF-κB has been shown to upregulate BMP2. BMP2 is a secreted protein that prior studies have shown is important in cellular differentiation and organogenesis, although its effects on fibroblast are unclear. Additionally, prior studies have shown that BMP2 is upregulated following $PM_{2.5}$ exposure in other cell types, such as bronchial epithelial cells. We set out to determine the effect of $PM_{2.5}$ exposure on BMP2 expression and secretion in fibroblasts, and to determine the role of BMP2 on $PM_{2.5}$-induced myofibroblast differentiation. We hypothesize that exposure to $PM_{2.5}$ will increase BMP2 and that this plays a major role in promoting myofibroblast differentiation. BMP2 transcript and protein levels following $PM_{2.5}$ exposure were assessed in fibroblasts, and BMP2 signaling experimentally manipulated by adding exogenous BMP2 protein to increase signaling or adding an inhibitory protein or siRNA to quench signaling. We found that $PM_{2.5}$ exposure promoted a dose-dependent increase in BMP2 transcript and secreted protein in pulmonary fibroblasts. Furthermore, treatment with exogenous BMP2 promoted myofibroblast differentiation, while blocking endogenous BMP2 with an siRNA or inhibitory protein, also promoted myofibroblast differentiation. This suggests that while too much BMP2 signaling induces myofibroblast differentiation, a baseline level is necessary to maintain homeostasis and prevent aberrant activation.

3.2 Introduction

In the previous chapter, we demonstrated that repeated exposure to low concentrations of $PM_{2.5}$ caused an increase in myofibroblast differentiation by signaling through NF-κB. However, the mechanism by which NF-κB promotes myofibroblast differentiation is unknown. NF-κB is a transcription factor that modulates the expression of a myriad of genes, including cytokines and other inflammatory molecules. In this Aim, we sought to examine one such cytokine and target of NF-κB[30], bone morphogenic protein (BMP) 2.

BMPs are secreted cytokines in the TGFβ superfamily of proteins. They include a group of molecules (BMP1-18) that play pivotal roles in development and organ morphology[68]. BMPs have been shown to be critical during body axis and limb formation[109, 110, 111, 112], as well as renal[113], digestive[114], neuronal[115, 116], reproductive[117] and skeletal development[70, 118]. While much remains to be elucidated about the exact role of BMPs in development, it is understood that BMPs are important secreted proteins that in conjunction with other mediators and signaling pathways, such as FGFs, Notch, and Wnt–β-catenin signaling, direct growth and orientation during early development. Specifically, BMPs contribute to the formation of the ectoderm, mesoderm, and endoderm[119, 120, 121, 122]. As their name implies, BMPs were initially discovered for their ability to induce bone and cartilage formation, but they have also been shown to be a significant mediator for cell and tissue differentiation in a variety of different organs well into adulthood[70]. For example, BMP signaling promotes epithelial to mesenchymal transition (EMT)[69], a process in which epithelial cells acquire a mesenchymal phenotype. EMT is considered important in the development of diseases like cancer and fibrosis[123, 124]. BMPs ability to modulate differentiation in different cell types has led us to hypothesize that they may be important in the mechanism by which $PM_{2.5}$ induces myofibroblast differentiation.

While BMPs as a family are a diverse group of molecules, certain BMPs share similar functions. BMP 2, -4, and-7 signal through the same receptor and demonstrate overlapping, redundant functions[125, 126]. BMP2 exerts its actions by signaling through two putative receptors, BMPRI and BMPRII. Activation of the BMP receptors leads to the downstream phosphorylation and recruitment of SMADs 1/5/8. The activated SMADs then form a complex with SMAD4 and translocate into the nucleus where they trigger changes in gene expression[127]. This SMAD-dependent pathway is thought of as BMPs canonical signaling pathway. In addition to the SMAD-dependent pathway, there is a SMAD-independent pathway that signals through MAPK and activates p38 and JNK[68]. The SMAD independent pathway is known as the non-canonical signaling pathway.

The fact that BMP2 signals through multiple pathways indicates that it may have diverse actions depending on the context. The ability of BMP2 to recruit SMADs 1/5/8 would imply that it opposes the profibrotic SMADs 2/3 that are typically activated by TGF-β1[127]. Perhaps it is unsurprising then, that BMP2 has been shown to have antifibrotic effects in many organ systems including the pancreas[128, 129], kidneys[130, 131] and the liver[132]. Additionally, *in vitro* studies of primary human lung fibroblasts demonstrated that BMP2 opposed the profibrotic effects of Endothelin-1[133].

Conversely, BMP2 has also been shown to promote profibrotic changes at times. This has been shown in mouse kidney cells, in which BMP2-MAPK activation promoted the expression of fibronectin, collagen I, and αSMA[72]. BMP2 differentiates renal mesenchymal progenitor cells into myofibroblasts through NOX4[71]. Others have shown that BMP2 and BMP7 dose dependently increase ACTA2 transcript levels in vascular smooth muscle and that BMP7 does so through increased nuclear localization of the myocardin-related transcription factors (MRTF-A

and MRTF-B) to a smooth muscle alpha-actin promoter[134]. BMP2 may be imparting some of these profibrotic effects through activation of the p38-MAPK pathway[135, 136, 137], which has been previously shown to promote myofibroblast differentiation[138, 139].

Regulation of BMP2 has been shown to occur via NF-κB. The BMP2 gene has two putative NF-κB response elements in its promoter[108]. Investigators have also shown that NF-κB regulates BMP2 expression in chondrocytes[108]. Given the link between NF-κB and BMP2, as well as BMP2s ability to impact myofibroblast differentiation, we hypothesize that BMP2 may be a significant contributor to the increase in myofibroblast differentiation following repeated, low concentration $PM_{2.5}$ exposure. Here, we sought to determine the effects of BMP2 on fibroblast biology in general and myofibroblast differentiation specifically, as well as whether $PM_{2.5}$ exposure affects the expression of BMP2 in pulmonary fibroblasts. We hypothesize that BMP2 promotes myofibroblast differentiation and that its expression and production are upregulated by $PM_{2.5}$.

3.3 Methods

Cell Culture

Normal primary human lung fibroblasts (CCL210), obtained from American Type Culture Collection (Manassas, Virginia), were cultured in DMEM (Thermo Fisher Scientific, Waltham, Massachusetts) supplemented with 10% FBS and 1% penicillin/streptomycin and maintained in a 37° C incubator with 5% CO_2. Additionally, some experiments also utilized lung epithelial cells. For these experiments, we obtained a549 cells (once again, from American Type Culture Collection) and cultured them in the same conditions as the pulmonary fibroblasts.

$PM_{2.5}$ Collection and Preparation

Cells were treated with two sources of $PM_{2.5}$. $PM_{2.5}$ sources were obtained from the National Institute of Standards and Technology (SRM 1649b, Gaithersburg, Maryland; henceforth referred to as NIST-$PM_{2.5}$) and from collaborators at the Peking University School of Public Health in Beijing (referred to hereafter as Beijing-$PM_{2.5}$). NIST-$PM_{2.5}$ was chosen as it is well-characterized and has been studied by many investigators over many years. It was originally collected from Washington, D.C. in 1976. NIST-$PM_{2.5}$ is shipped as a solid and was solubilized in double distilled water by sonication (VWR, model no. 97043–968, VWR International, Radnor, Pennsylvania, USA).

Collaborators in Beijing collected the Beijing-$PM_{2.5}$ from a manual sampler on the rooftop of the Peking University School of Public Health, a location with high levels of traffic, diesel, and urban-related pollution. Emfab filters (90 mm), consisting of borosilicate fibers reinforced with woven cloth and bonded with polytetrafluoroethylene (TX40HI20WW, part #7234, Pall Company, Beijing Office, Beijing, China) were utilized to collect Beijing-$PM_{2.5}$. These filters were replaced every 24 hours. Filters were then folded in half and shipped to the US in sterile secure packaging. Upon arrival they were stored at −20° C until extraction.

$PM_{2.5}$ was extracted from the filters as previously described[91]. Briefly, each filter was equilibrated in sterile conditions at constant humidity and room temperature for 24 hours before extraction. Prior to extraction, each filter was weighed on a microbalance (AC 100, Mettler-Toledo, Columbus, OH). $PM_{2.5}$ was extracted by placing each filter face down in amber jars, wetting each filter with 20 ml of double distilled water, and then sonicating on ice at 15 min intervals for a total of 3 hours. After sonication, filters were kept in amber jars in sterile conditions at constant humidity and room temperature for 3 days to air-dry. After drying the filters were weighed on a microbalance. The average difference in weight before and after

extraction was used to calculate the concentration (mg/ml). Samples were aliquoted and stored for future use at −80° C.

Treatment of cells with $PM_{2.5}$

Treatment protocols used here are essentially the same as those used in the prior chapter. Restated briefly, the cells were plated at 2.5×10^5 cells/well in 6-well plates prior to treatment. The following day, medium was removed, and the cells were incubated for approximately 24 hours in serum-free DMEM. Cells were then treated with various concentrations of $PM_{2.5}$ (0.01 – 30 μg/cm^2), diluted in DMEM for the indicated times. For certain experiments, cells were treated with repeated doses of $PM_{2.5}$. In these instances, medium was removed before each dose, cells were washed with PBS, and fresh medium with $PM_{2.5}$ (0.01 – 10 ug/cm^2) was added. Control cells in these repeated exposure experiments had medium replaced with DMEM alone. After the exposure period(s), cells were harvested for RNA or protein analysis. Additionally, the supernatant was also collected. In some experiments, the cells were preincubated with exogenous Noggin (R&D Systems, Minneapolis, MN, USA), a protein that binds and inhibits BMPs, for 30 m prior to each $PM_{2.5}$ exposure, or pretreated with a BMP2 siRNA vs control siRNA 24 hours prior to the first $PM_{2.5}$ exposure. Noggin was chosen because it is a well-established inhibitor of BMP signaling[140].

Treatment of cells with exogenous BMP2

Fibroblasts were plated at 2.5×10^5 cells/well in 6-well plates prior to treatment. The following day, medium was removed, and the cells were incubated for approximately 24 hours in serum-free DMEM. Cells were then treated with various concentrations of exogenous BMP2 (20ng/ml, 100ng/ml, or 200ng/ml) (R&D Systems, Minneapolis, MN, USA) diluted in DMEM for the 72 hours. After the treatment period, cells were harvested for RNA or protein analysis.

RNA Extraction and quantitative real-time PCR

RNA was isolated from the fibroblasts using Trizol (Catalog Number 15596018, Invitrogen, Carlsbad, CA, USA). RNA was quantified with a Nanodrop Spectrophotometer (NanoDrop 2000, Nanodrop Technologies LLC, Thermo Fisher Scientific, Wilmington, DE, USA) and stored at -80 °C until processing. RNA was reverse-transcribed by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's recommendations. Quantitative real-time PCR was performed on the resulting cDNA using SYBER green PCR Master Mix (Applied Biosystems) on a StepOne Real-time PCR System (Applied Biosystems). The presence of a single peak on the melting curve verified primer specificity. The fold change in target gene expression was calculated by the ΔΔCt method, using β-actin as the endogenous control. Table 3.1 lists the primers used.

Immunoblot Analysis

Fibroblasts were lysed in lysis buffer (PBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN) and Phosphatase Inhibitor I and II Cocktails (EMD Millipore, Billerica, MA). In some experiments, protein levels in the supernatant were assessed as well. The DC Protein Assay (5000111, Biorad, Hercules, CA) was used to determine protein concentration and equal protein was loaded onto either 8% or 10% tris-glycine gels, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were blocked with 5% bovine serum albumin before being probed overnight at 4° C with the antibodies listed in Table 3.2. Membranes were then incubated with appropriate secondary antibody conjugated to horseradish peroxidase (Cell Signaling) for 1 hour at room temperature before developing with enhanced chemiluminescent

reagent (GE Healthcare, Pittsburgh, PA). For all protein bands, densitometry was analyzed by Image J Software (NIH, Bethesda, MD) and normalized to α-tubulin or GAPDH.

Statistical Analysis

The statistical significance of the quantitative real-time PCR data was determined by performing an analysis of variance (ANOVA) in Prism (GraphPad Prism Software v7, La Jolla, CA). Western Blot data was quantified by densiometric methods and significance tested via ANOVA. Dunnett's test was used for post hoc testing in both instances.

3.4 Results

Treatment of fibroblasts with exogenous BMP2 stimulated myofibroblast differentiation

As previously alluded, literature on the effects of BMP2 in the lung are conflicting. To determine the effect BMP2 specifically has on lung fibroblasts, we treated normal, quiescent lung fibroblasts with varying doses of recombinant human BMP2 and analyzed levels of αSMA mRNA and protein (Figure 3.1A). We found that in response to exogenous BMP2 treatment, αSMA mRNA and protein levels were elevated (Figure 3.1B – 3.1D). These data indicate that BMP2 may be an important cytokine that promotes, rather than inhibits, myofibroblast differentiation.

$PM_{2.5}$ exposure promotes an increase in BMP2

We next sought to determine whether BMP2 expression is affected in response to $PM_{2.5}$ exposure. We previously showed in Aim 1 that myofibroblast differentiation occurs with repeated exposure to $PM_{2.5}$. Here, we assessed levels of BMP2 transcript after exposing cells to $PM_{2.5}$ twice or every day for six days $PM_{2.5}$ (Figure 3.2E & 3.2F, respectively). We found that BMP2 transcript was upregulated in response to repeated and daily exposures (Figure 3.2A & 3.2B, respectively). We next sought to determine if $PM_{2.5}$ increases BMP2 even after a single

exposure (Figure 3.2G). We found that a single $PM_{2.5}$ exposure caused a dose dependent increase in BMP2 mRNA in lung fibroblasts. The increase in BMP2 was observed in fibroblasts treated with either NIST-$PM_{2.5}$ or Beijing-$PM_{2.5}$ (Figure 3.2C & 3.2D, respectively), indicating that induction of BMP2 transcript may be generalizable to urban PM from different geographic sources. While the upregulation of BMP2 transcript following repeated $PM_{2.5}$ exposure was greater than the upregulation that followed a single exposure, it was not as dramatic a difference as there was in the induction of αSMA following single and repeated exposures.

$PM_{2.5}$ exposure does not promote an increase in other BMP/BMP-related genes

As previously stated, BMP2 is just one of many proteins in the BMP family. Certain subsets of BMPs often have overlapping functions and can be regulated in tandem[125, 126]. Given the clear increase observed in BMP2 transcription, we measured levels of other BMP and BMP related genes in our $PM_{2.5}$ exposed fibroblasts. We found no increase in BMP4 (Figure 3.3A) and were unable to detect BMP7 or BMP9 in our samples; indicating that $PM_{2.5}$ uniquely upregulates BMP2 and not other common members of the BMP family of genes in pulmonary fibroblasts. Additionally, we found that Gremlin 1, a BMP antagonist, was down regulated following $PM_{2.5}$ exposure (Figure 3.3B). Notably, the doses at which Gremlin 1 was downregulated corresponded to the doses at which BMP2 was upregulated. Finally, the expression of BMPER, another protein that modulates BMP signaling was also assessed and this was not altered in a statistically significant manner following $PM_{2.5}$ exposure (Figure 3.3C).

Treatment of fibroblasts with $PM_{2.5}$ resulted in increased secretion of BMP2 protein

To confirm that the increase in BMP2 transcript was reflected by increases in protein expression, we performed a Western blot for BMP2 in fibroblasts treated with increasing doses

of $PM_{2.5}$ (Figure 3.4A). Given that BMP2 is a secreted protein, we examined expression of BMP2 in Western blots of both total cell lysates and in cellular supernatants.

While BMP2 was not detected in the cell lysates themselves, it was detected in the secreted supernatant of the cells. BMP2 secretion increased in a dose dependent fashion with $PM_{2.5}$ (Figure 3.4B). Interestingly, in response to high doses of $PM_{2.5}$ (30 μg/cm^2), a second band corresponding to a lighter molecular weight was also detected. This lighter band also appeared in the positive control condition (recombinant BMP2), leading us to surmise that this may be BMP2 as well, most likely the BMP2 monomer. Overall, these data suggest that $PM_{2.5}$ exposure causes an upregulation of BMP2 transcript that in turn leads to an increase in secreted BMP2.

Fibroblasts synthesize BMP2 to levels comparable to alveolar epithelial cells following $PM_{2.5}$ exposure

BMP2 can be synthesized by other cells in the lung, including epithelial cells. To determine the relative levels of BMP2 synthesized by fibroblasts compared to epithelial cells, we assayed levels of secreted BMP2 in fibroblasts and A549 cells, an alveolar epithelial cell line, by immunoblot (Figure 3.5). We detected BMP2 protein in the conditioned medium of both fibroblasts and epithelial cells. While epithelial cells express higher levels of BMP2 compared to normal fibroblasts at baseline, in the setting of $PM_{2.5}$ exposure, fibroblasts demonstrated an increase in BMP2 expression to levels comparable to or even greater than epithelial cells. This implies that during homeostasis, epithelial cells may be the predominant source of BMP2 within the alveolar space, but after exposure to $PM_{2.5}$, fibroblasts are also important cells that synthesize and secrete BMP2.

Treatment with BMP2 siRNA has minimal effect on αSMA expression

We previously observed that treating fibroblasts with BMP2 promoted myofibroblast differentiation. This however, required exogenous administration of BMP2 at concentrations that may be higher than what fibroblasts endogenously synthesize, even when BMP2 is upregulated by $PM_{2.5}$. To determine whether endogenous synthesis of BMP2 following $PM_{2.5}$ exposure induces myofibroblast differentiation, we silenced BMP2 with siRNA and examined the effects of $PM_{2.5}$ on αSMA expression. In our exposure protocol (Figure 3.6A), a single treatment of BMP2 siRNA was able to block the $PM_{2.5}$ induced increase in BMP2 transcript that was previously observed after 72 hours (Figure 3.6B). When BMP2 siRNA was given prior to repeated low concentration $PM_{2.5}$ exposure, the increase in αSMA expression was not inhibited in the presence of BMP2 siRNA. (Figure 3.6C/3.6D).

Although $PM_{2.5}$ causes an increase in αSMA expression at low concentrations, we have also consistently observed that this does not occur at higher concentrations of $PM_{2.5}$. This was similarly observed in cells treated with control siRNA (Figure 3.6E). However, treatment of cells with BMP2 siRNA resulted in an increase in αSMA at baseline, and this increase was unaffected by the presence of $PM_{2.5}$. These data indicate that silencing BMP2 expression results in an increase in total αSMA expression independent of the effects of $PM_{2.5}$.

Treatment with Noggin rescues αSMA protein expression at high doses

Because we observed that silencing BMP2 did not inhibit $PM_{2.5}$-induced increases in αSMA and instead increased basal levels of αSMA, we hypothesized that endogenous BMP2 might instead serve as a brake on myofibroblast differentiation. To use an alternative approach to inhibiting BMP signaling, we treated cells with Noggin, an inhibitor of BMP signaling that previously was shown to inhibit BMP2 as well as other BMP proteins[140] (Figure 3.7A). In control cells without Noggin, repeated $PM_{2.5}$ exposure increased αSMA at low concentrations,

but not at higher concentrations. Cells pre-treated with Noggin, however, demonstrated a trend toward increases in αSMA at both high and low concentrations of $PM_{2.5}$ (Figure 3.7B/3.7C). Together, these data demonstrate that inhibition of BMP2 – with either BMP2 siRNA or Noggin – results in increased levels of αSMA. These data imply that although treatment with exogenous BMP2 results in increased αSMA expression, endogenous BMP2 might inhibit αSMA and could explain why treatment with higher concentrations of $PM_{2.5}$, which typically increases BMP2 levels, does not lead to the same induction of αSMA as that observed with lower concentrations of $PM_{2.5}$. When combined with our data on the dose response of BMP2 expression to $PM_{2.5}$ exposure, these data convincingly demonstrate that BMP2 is not the downstream mechanism being acted on by NF-κB following repeated low concentration $PM_{2.5}$ exposure.

3.5 Discussion

Here, we show that exposure to $PM_{2.5}$ increased BMP2 in a robust, dose-dependent fashion. This increase was at both the mRNA and protein level and resulted in increased BMP2 secretion. The increase was specific to BMP2 and not observed in other BMP family members. Treatment with exogenous BMP2 promoted myofibroblast differentiation. However, synthesis of endogenous BMP2 appeared to inhibit, rather than increase, myofibroblast markers; and the ability of $PM_{2.5}$ to induce myofibroblast differentiation at low concentrations was not due to BMP2.

Prior literature had shown that $PM_{2.5}$ induces BMP2 expression, but these studies were performed in other cell types and none have examined its ability to affect synthesis of BMP2 in fibroblasts. Global gene expression profiling of human bronchial epithelial cells exposed to $PM_{2.5}$ revealed a statistically significant increase in BMP2 expression[107]. Human umbilical vein endothelial cells upregulate BMP2 in response to exposure to carbon black, a component of

diesel exhaust[141]. $PM_{2.5}$ exposure *in vivo* caused an Nrf2 dependent increase in BMP2 expression in the hearts of C57/B6 mice[142]. Here, we show in fibroblasts that BMP2 transcript and protein expression are increased in a reproducible, dose dependent fashion by $PM_{2.5}$. This occurred with a variety of exposure protocols and with the use of two independent sources of $PM_{2.5}$. Furthermore, by assessing levels of BMP2 in both alveolar epithelial cells and fibroblasts, we demonstrated that fibroblasts synthesize BMP2 after $PM_{2.5}$ exposure to levels comparable to epithelial cells. This indicates that the amount of BMP2 synthesized by fibroblasts after $PM_{2.5}$ exposure is biologically significant and likely important to lung homeostasis and repair.

We initially hypothesized that the increase in BMP2 was responsible for stimulating myofibroblast differentiation following the repeated, low dose $PM_{2.5}$ exposures that we performed in our prior Aim. While treatment of fibroblasts with exogenous BMP2 (Figure 3.1) supported this notion, additional experiments inhibiting endogenous BMP2 levels showed opposite effects. First, we noticed that $PM_{2.5}$ increased BMP2 only after cells were treated with a high concentration of $PM_{2.5}$, whereas increase in myofibroblast differentiation occurred only in response to repeated, low concentration exposures. Furthermore, we found that when we blocked BMP2 signaling with either an inhibitory protein (Noggin) or siRNA, neither Noggin nor BMP2 siRNA were able to inhibit the ability of low-concentration $PM_{2.5}$ to cause myofibroblast differentiation. These results suggest that BMP2 has opposite effects at different concentrations. At the concentrations present in our exogenous BMP2 experiments, BMP2 promoted myofibroblast differentiation, while at the concentrations present in our $PM_{2.5}$ exposure experiments, BMP2 inhibiting myofibroblast differentiation.

Interestingly, blocking BMP2 with Noggin or BMP2 siRNA at baseline resulted in increased levels of αSMA. This indicates that endogenous levels of BMP2 may serve as a brake

against myofibroblast differentiation. Notably, only low concentrations of $PM_{2.5}$ resulted in an increase in myofibroblast differentiation and levels of αSMA were not increased when cells were treated with higher concentrations of $PM_{2.5}$. These higher $PM_{2.5}$ concentrations mirrored the concentrations that were observed to cause an increase in BMP2 synthesis. Furthermore, when BMP2 signaling was blocked via Noggin or BMP2 siRNA, αSMA levels remained high regardless of the $PM_{2.5}$ concentration. These data imply that BMP2 may actually be exerting an inhibitory effect on myofibroblast differentiation in our $PM_{2.5}$ experiments.

One of our original goals was to elucidate the role that BMP2 had on fibroblast biology. Interestingly, we have data pointing us in two separate directions. On one hand, exogenous treatments with BMP2 clearly promoted an increase in αSMA expression and myofibroblast differentiation. On the other, silencing BMP2 or blocking BMP signaling did not block the increase in αSMA and in fact, demonstrated that endogenous levels of BMP2 may in fact inhibit myofibroblast differentiation. Prior research has shown that BMP2 can have different effects at different concentrations[143]. It's possible that the concentration of BMP2 used in the exogenous BMP2 treatment experiments differs considerably from the concentration secreted in response to $PM_{2.5}$ exposure. Our attempts to quantify BMP2 levels were unsuccessful, but that information could validate this hypothesis.

It is interesting to note, that similar, seemingly contradictory results have been found with BMP2 in the epithelial cell literature. Specifically, two independent studies looked at the effect of BMP signaling on the differentiation of alveolar epithelial type II to type I cells, with one group finding that BMP signaling inhibits differentiation[144], and another found that it promotes it[145]. This supports the idea that the overall effect of BMP signaling is highly dependent on dose and context.

In this chapter we established that $PM_{2.5}$ exposure promotes a significant dose-dependent increase in BMP2 transcript and protein levels. While this is a novel finding in pulmonary fibroblasts, the effect that increased BMP2 signaling would have was unclear. Our studies showed that the effects of BMP2 on fibroblast biology were context and likely dose dependent. Exogenous treatments promoted myofibroblast differentiation, while a comparison of the dose-response curves and the Noggin/siRNA experiments suggested a more antifibrotic effect for BMP2. We believe that this apparent contradiction is due to BMP2's biphasic dose effect. Specifically, that baseline levels of BMP2 signaling promote homeostasis in fibroblasts. When this signaling is disrupted, the cells become more vulnerable to environmental insult. However, increases in BMP2 levels beyond a certain point may become deleterious. Further work quantifying the levels of BMP2 following $PM_{2.5}$ exposure, as well as determining the specific signaling pathways that BMP2 is acting on (i.e. canonical SMAD vs. non canonical MAPK) will help shed further light on these results. Additionally, the downstream mechanism by which NF-κB promoted myofibroblast differentiation following low concentration, repeated $PM_{2.5}$ exposure remains unknown and is a potential target for future investigation.

3.6 Tables

Table 3.1: Primers utilized in Aim 2

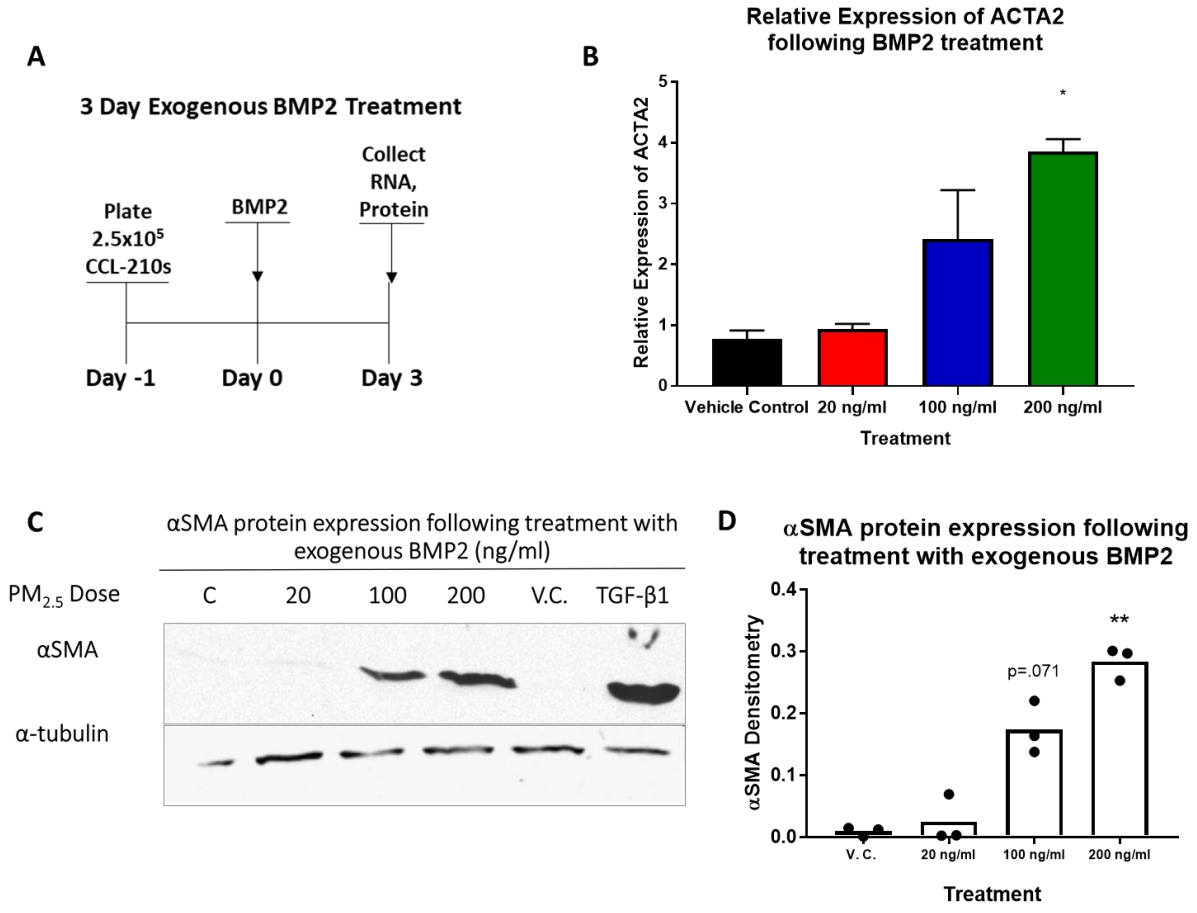
Primer	Sequence
β-actin	Forward TAGTTGCGTTACACCCTTTC Reverse GCACGAAGGCTCATCATT
ACTA2	Primers with specific probes used to amplify and detect ACTA2 (Hs00426835_g1) were obtained from Applied Biosystems
BMP2	Forward TGTATCGCAGGCACTCAGGTCA Reverse CCACTCGTTTCTGGTAGTTCTTC
BMP4	Forward AGCATGTCAGGATTAGCCGA Reverse TGGAGATGGCACTCAGTTCA
BMP7	Forward GGCAGGACTGGATCATCG Reverse AAGTGGACCAGCGTCTGC
BMP9	Forward CCTGGGCACAACAAGGAC Reverse CCTTCCCTGGCAGTTGAG
GREM1	Forward GCCCTCGGGAGCCACAAACC Reverse GCAGCAGGAGTCGCGGTGAG
BMPER	Primers with specific probes used to amplify and detect BMPER (Hs.PT.58.3315719) were obtained from Applied Biosystems

Table 3.2: Antibodies utilized in Aim 2

Protein	Dilution	Company
GAPDH	1:1000	Santa Cruz, Dallas, TX, USA
α-tubulin	1:4000	Sigma-Aldrich, St. Louis, MO, USA
αSMA	1:1000	Dako Brand, Agilent Technologies, Santa Clara, CA, USA
BMP2	1:500	R&D Systems, Minneapolis, MN, USA

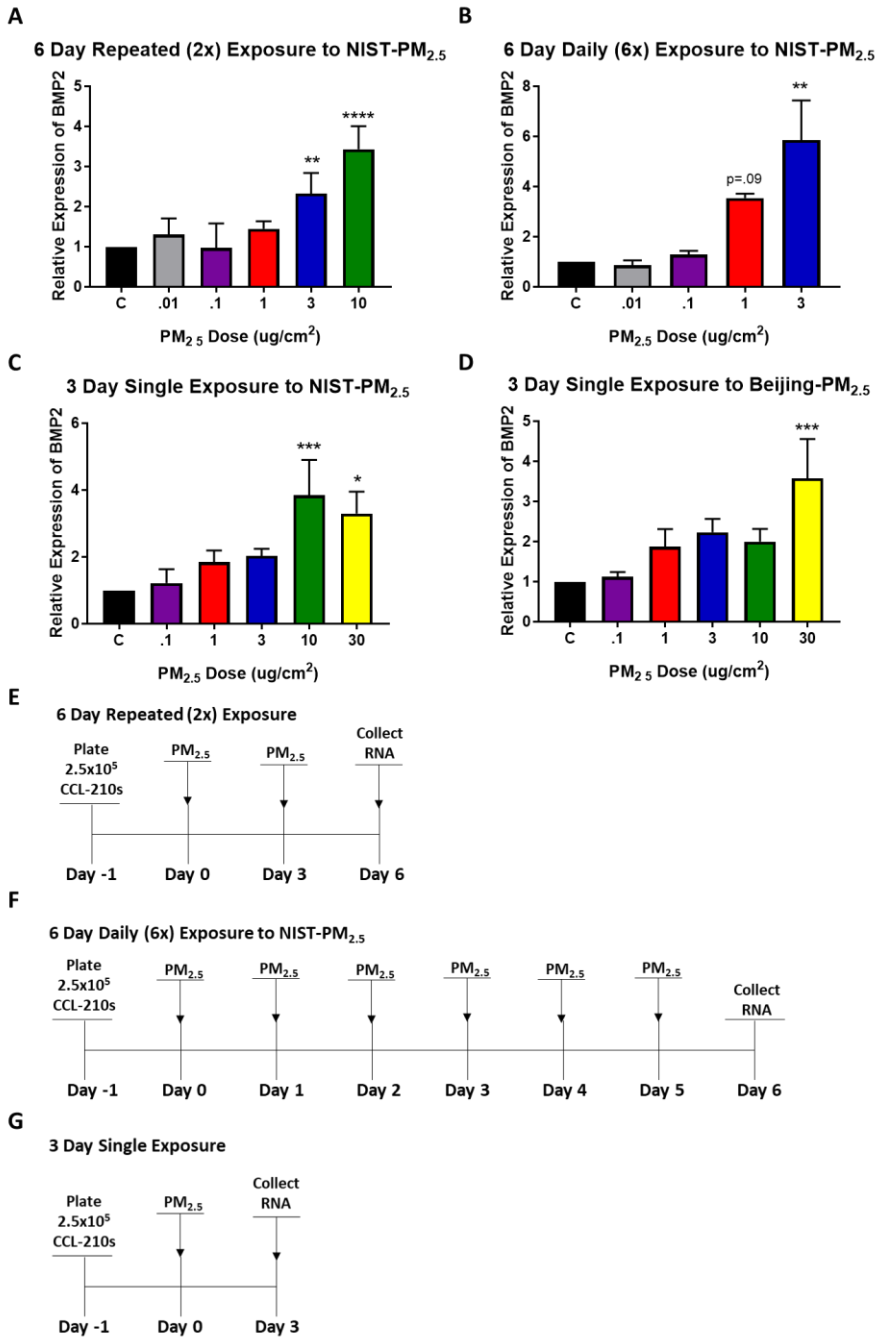
3.7 Figures

Figure 3.1: Treatment of fibroblasts with exogenous BMP2 stimulated myofibroblast differentiation



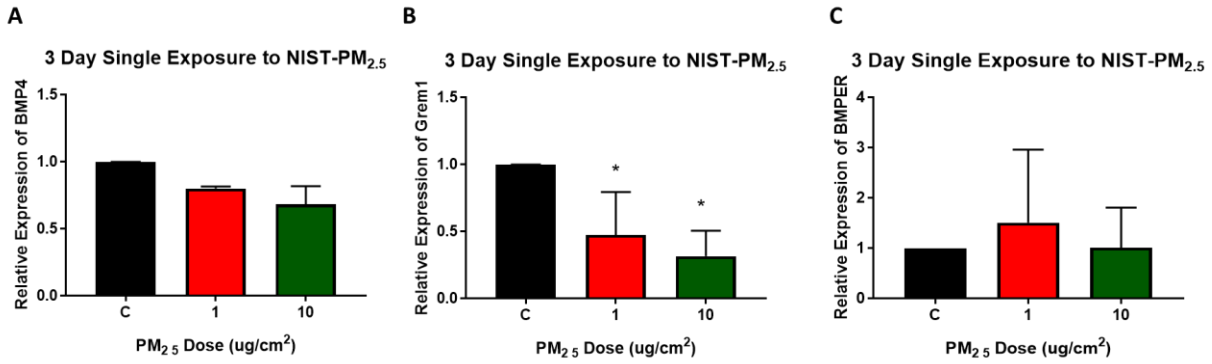
Legend – CCL-210 fibroblasts were treated with exogenous BMP2 A) A wire diagram depicting the 3 day exogenous BMP2 treatment that the CCL-210 fibroblasts received. B) ACTA2 transcript levels following 3 day treatment with exogenous BMP2; assessed via rt-qPCR (housekeeping gene – β-actin). C) Representative immunoblot for αSMA protein level following 3 day treatment with exogenous BMP2. D) Densitometry for αSMA protein levels following 3 day treatment with exogenous BMP2. Abbreviations: V.C. – Vehicle Control. *p<0.05, **p<0.005 relative to control.

Figure 3.2: $PM_{2.5}$ exposure promotes an increase in BMP2 transcript



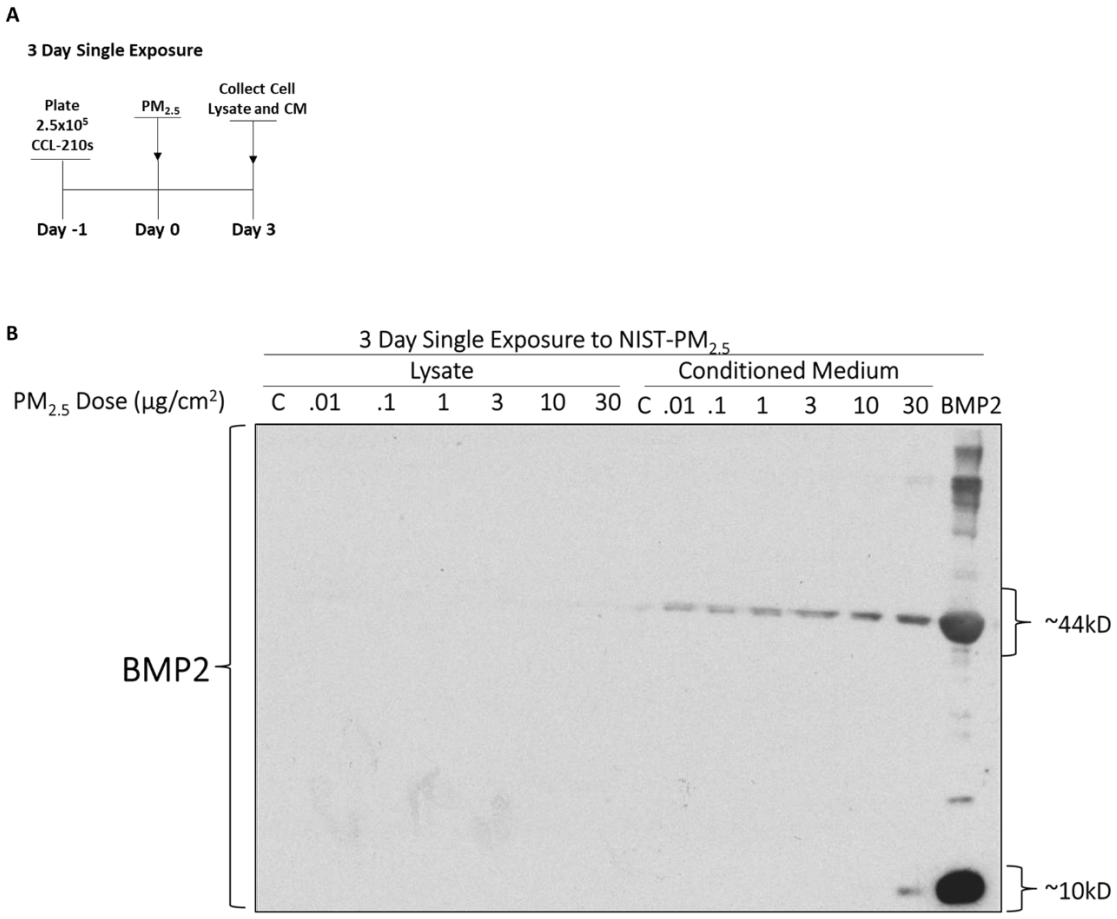
Legend – BMP2 transcript levels following various $PM_{2.5}$ exposures (A-6 day repeated NIST-$PM_{2.5}$, B-6 day daily NIST-$PM_{2.5}$, C-3 day single NIST-$PM_{2.5}$, D-3 day single Beijing-$PM_{2.5}$) as assessed by rt-qPCR (housekeeping gene – β-actin). The protocols for the 6 day repeated, 6 day daily, and 3 day single exposures are shown in (E), (F), and (G), respectively. *$p<0.05$, **$p<0.005$, ***$p<0.0005$, ****$p < 0.00005$ relative to control.

Figure 3.3: $PM_{2.5}$ exposure does not promote an increase in other BMP/BMP-related genes



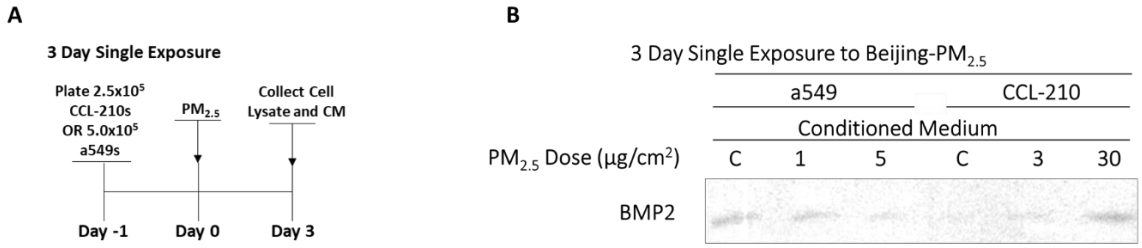
Legend – Assessment with rt-qPCR (housekeeping gene – β-actin) showed levels of BMP4 (A), GREM1(B), and BMPER (C) transcript following 3 day single exposure to NIST-$PM_{2.5}$. *$p<0.05$ relative to control.

Figure 3.4: Treatment of fibroblasts with $PM_{2.5}$ resulted in increased secretion of BMP2 protein



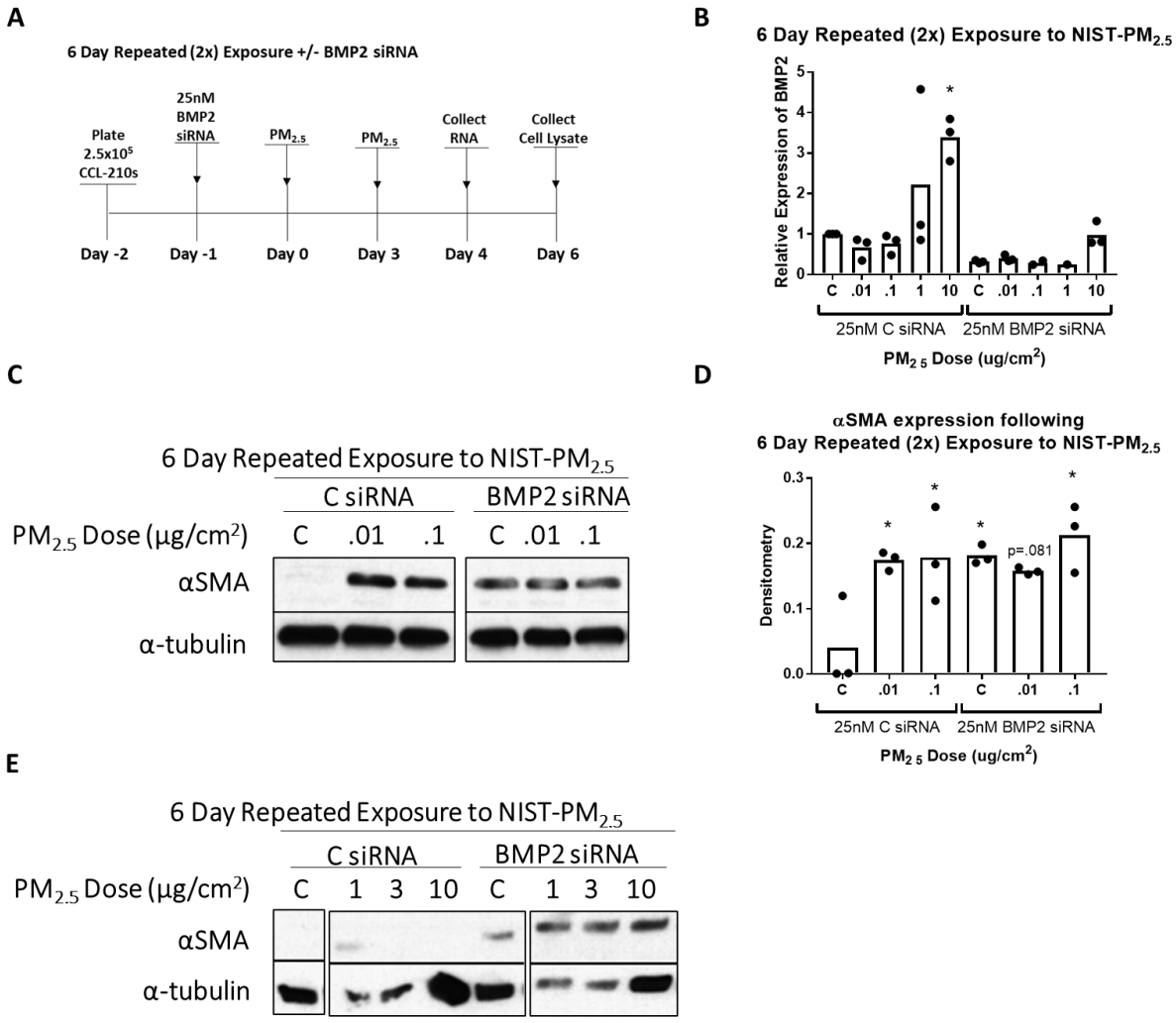
Legend – BMP2 protein levels following $PM_{2.5}$ exposure. A) A wire diagram depicting the 3 day single exposure protocol in which CCL-210 cells were treated with NIST-$PM_{2.5}$. (B) Representative immunoblot of BMP2 expression in fibroblasts exposed to NIST-$PM_{2.5}$ (n=3).

Figure 3.5: Fibroblast synthesize BMP2 to levels comparable to alveolar epithelial cells following $PM_{2.5}$ exposure



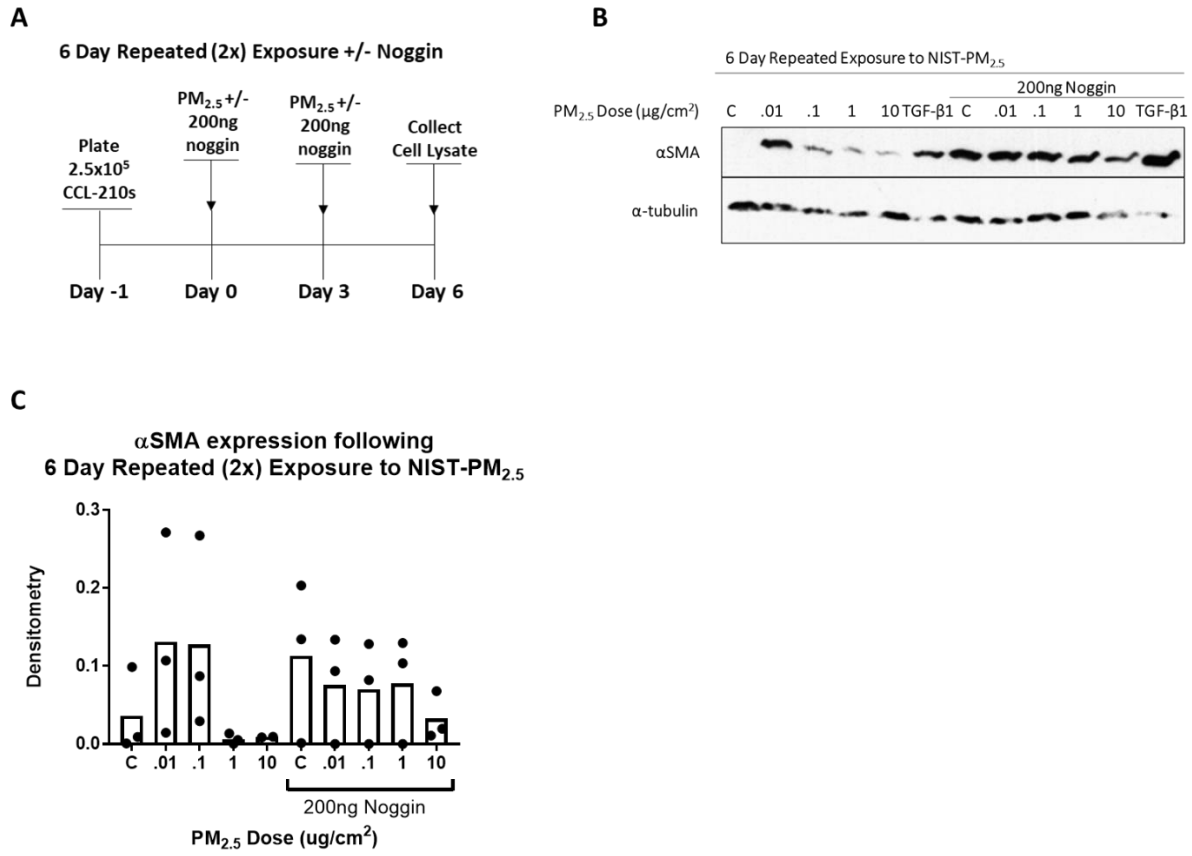
Legend – BMP2 production in alveolar epithelial cells and fibroblasts following $PM_{2.5}$ exposure. A) A wire diagram depicting the 3 day single exposure protocol in which CCL-210 and a549 cells were treated with Beijing-$PM_{2.5.}$ (B) Immunoblot of BMP2 expression in supernatant of fibroblasts and alveolar epithelial cells exposed to Beijing-$PM_{2.5.}$

Figure 3.6: Treatment with BMP2 siRNA has minimal effect on αSMA expression



Legend – αSMA levels after blocking BMP2 expression with a BMP2 siRNA. A) A wire diagram depicting the 6 day repeated exposure protocol +/- BMP2 siRNA treatment. B) BMP2 transcript levels following treatment with BMP2 siRNA; assessed via rt-qPCR (housekeeping gene – β-actin). C) Representative immunoblot for αSMA protein levels following 6 day repeated exposure to low concentrations of NIST-$PM_{2.5}$ +/- BMP2 siRNA. D) Densitometry for αSMA protein levels following 6 day repeated exposure to low concentrations of NIST-$PM_{2.5}$ +/- BMP2 siRNA. E) Representative immunoblot for αSMA protein levels following 6 day repeated exposure to high concentrations of NIST-$PM_{2.5}$ +/- BMP2 siRNA. *$p<0.05$ relative to control.

Figure 3.7: Treatment with Noggin rescues αSMA protein expression at high doses



Legend – αSMA levels after blocking BMP2 signaling with Noggin. A) A wire diagram depicting the 6 day repeated exposure protocol +/- Noggin treatment. B) Representative immunoblot for αSMA protein levels following 6 day repeated exposure to NIST-$PM_{2.5}$ +/- Noggin. C) Densitometry for αSMA protein levels following 6 day repeated exposure to NIST-$PM_{2.5}$ +/- Noggin.

Chapter 4 The Effect of $PM_{2.5}$ Exposure on DNA Methylation and Cytokine Secretion in Pulmonary Fibroblasts

4.1 Introduction

Although our work in previous chapters has shown that $PM_{2.5}$ promotes myofibroblast differentiation (through NF-κB signaling) and induces synthesis and secretion of BMP2, $PM_{2.5}$ likely causes a myriad of changes in fibroblasts through diverse mechanisms. One way that environmental insults can cause persistent changes to affect disease susceptibility is through epigenetic modifications, such as DNA methylation. DNA methylation is process in which methyl groups are added to DNA. These additions often have significant impacts on gene expression[146, 147]. Prior work has shown that alterations in DNA methylation levels are correlated to negative health outcomes, including aging, cancer and fibrosis[148, 149]. Furthermore, $PM_{2.5}$ exposure has been shown to alter DNA methylation levels in biologically significant ways, specifically, $PM_{2.5}$ exposure has been shown to cause changes in DNA methylation that are correlate with increased incidence of lung cancer[81], and asthma[82], as well as decreases in markers of healthy aging[78, 80]. Additionally, methylation of the genes THY1, PTGER2, CDKN2B, and KCNMB1 have been shown to promote myofibroblast differentiation[150], and genome-wide methylation patterns in IPF fibroblasts have been shown to be different than methylation patterns of normal fibroblasts[151]. Finally, given the fact that repeated exposure to $PM_{2.5}$ was necessary for $PM_{2.5}$ to induce myofibroblast differentiation, we hypothesize that epigenetic changes may be occurring that prime cells for further change with subsequent exposure.

Fibroblasts are generally considered structural cells that only secrete matrix proteins that serve as scaffolds, but increasingly, they are also recognized to participate in immunomodulatory roles and secrete other factors, including cytokines. $PM_{2.5}$ has been shown to induce epithelial cells to secrete tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-1β, and intercellular adhesion molecule 1 (ICAM-1)[152, 153, 154, 155]. In fibroblasts, we had previously shown in Aim 2 that $PM_{2.5}$ causes the secretion of one such cytokine, BMP2. Here, we sought to determine if $PM_{2.5}$ causes the secretion of other cytokines in pulmonary fibroblasts.

Taken together, these experiments will provide information about additional ways that $PM_{2.5}$ exposure can affect fibroblast biology. Specifically, our goal in this chapter is to describe the effect of $PM_{2.5}$ exposure on global DNA methylation levels, and cytokine secretion profiles in pulmonary fibroblasts. This descriptive information will aid in the generation of new hypotheses and inform the direction of future research.

4.2 Methods

Methylation Analysis

Normal primary human lung fibroblasts (CCL210, American Type Culture Collection, Manassas, Virginia) were cultured as they were in Aim 1 and 2, and plated at 2.5×10^5 cells/well in 6-well plates overnight. Medium was subsequently removed, and the cells were incubated for approximately 24 hours in serum-free DMEM. Fibroblasts were then treated with various concentrations of NIST-$PM_{2.5}$ (3 or 30 μg/cm^2) for 72 hours. The collection and preparation of NIST-$PM_{2.5}$ is described in Aim 1 and 2. We performed three flights of replicate experiments. For each experiment, fibroblasts were treated for 72 hours with either no-$PM_{2.5}$ control, or NIST-$PM_{2.5}$ at a concentration of 3 μg/cm^2 or 30 μg/cm^2. After the exposure period, the DNA was

extracted using the DNeasy kit (Qiagen, Hilden, Germany) per manufacturer's protocol. The methods for the methylation analysis are shown in Figure 4.1A.

After extraction, DNA was sent to the University of Michigan Epigenomics Core for quantitation using the Qubit high sensitivity dsDNA assay (Invitrogen, Waltham, MA, USA), and quality assessment with the TapeStation genomic DNA kit (Agilent, Santa Clara, CA, USA). 250ng of each sample was bisulfite converted with Zymo's EZ DNA Methylation kit and using the manufacturer's incubation parameters specific for Illumina methylation arrays (Zymo Research, Irvine, CA, USA). After cleaning, samples were then sent to the University of Michigan DNA Sequencing Core for hybridization to the Infinium MethylationEPIC BeadChip array, washing, and scanning, according to the manufacturer's instructions (Illumina, San Diego, CA, USA).

Bioinformatic and GO Analysis

Snakemake (Köster and Rahmann, 2012) was utilized by the Epigenomics Core to manage the bioinformatics workflow in a reproducible manner. Raw red/green IDAT files were read into R using the minfi Bioconductor package (Fortin *et al.,* 2017) (v1.28.3). Initial quality control based on detection of p-values and signal intensity was performed using the ENmix Bioconductor package (Xu *et al.,* 2017) (v1.18.1). In particular, if a probe had detection p-value < 0.05 in more than 5% of samples it was removed. Similarly, if a sample had more than 5% of probes with detection p-value < 0.05, then that sample was removed. In this case 0 probes were removed.

After filtering to ensure quality, a series of corrections and normalizations occurred. Probe intensities were background-corrected using out-of-band Infinium I intensities and dye-

corrected using the RELIC algorithm (Xu *et al.*, 2017). Next, inter-array normalization was performed by separately quantile normalizing methylated and unmethylated intensities for Infinium I or II probes (Xu *et al.*, 2017). Then, probe-type biases were corrected for with the beta-mixture quantile normalization method (BMIQ) (Teschendorff *et al.*, 2013). Finally, any probe within 2bp of a SNP was removed, as were known cross-hybridizing probes (Peters *et al.*, 2015, Pidsley *et al.* (2016)).

To assess the impact of $PM_{2.5}$ exposure on DNA methylation in pulmonary fibroblasts, we compared low and high exposure groups to control, as well as low exposure and high exposure groups directly. For each comparison we used the limma R Bioconductor package (v3.38.3) to identify differentially methylated probes (DMPs) by fitting a linear model whose standard errors were then moderated using an empirical Bayes model (Ritchie *et al.*, 2015). The DMPs were then annotated to CpG island and genic annotations using the annotatr R Bioconductor package (v1.7.3) (Cavalcante and Sartor, 2017). The comparisons used in the analysis, as well as the thresholds used to determine DMPs among the comparisons were a p-value < 0.005 with differential methylation of 5% or greater. This p-value was used rather than FDR as FDR was too stringent a threshold given the low power of this exploratory study.

Annotating DMPs to specific genes produced a list of differentially methylated genes. We evaluated these genes using GO Enrichment analysis[156, 157]. This tool analyzes lists of genes and determines if a particular biological process has more genes associated with it on the lists than we expect by chance. Specifically, we ran our lists of differentially methylated genes through a GO Enrichment analysis and report the significantly enriched biological processes associated with our differentially methylated genes.

Luminex Experiment Design

For the Luminex array, normal primary human lung fibroblasts (CCL210, American Type Culture Collection, Manassas, Virginia) were cultured as they were in Aim 1 and 2, and plated at 2.5×10^5 cells/well in 6-well plates overnight. Medium was subsequently removed, and the cells were incubated for approximately 24 hours in serum-free DMEM. Fibroblasts were then treated with both Beijing-$PM_{2.5}$ and NIST-$PM_{2.5}$. The collection and preparation of both sources of $PM_{2.5}$ is described in Aim 1 and 2. We performed five flights of replicate experiments with NIST-PM, and three flights of replicate experiments with Beijing-PM. Within each flight, one sample was a control, one was exposed to a low concentration (1 μg/cm^2) of either Beijing-$PM_{2.5}$ or NIST-$PM_{2.5}$, and one was exposed to a high concentration (10 μg/cm^2) of either Beijing-$PM_{2.5}$ or NIST-$PM_{2.5}$. After the exposure period, the supernatant was collected for Luminex analysis. The methods for sample generation are shown in Figure 4.1B. The Luminex immunoassay was performed by collaborators at the North Campus Research Complex according to the manufacturer's instructions (Luminex, Austin, TX, USA). Statistical analysis of all analytes was carried out by two-way ANOVA with Bonferroni's multiple comparisons test to compare between experimental groups.

4.3 Results

$PM_{2.5}$ exposure promotes differential methylation in pulmonary fibroblasts

To determine how $PM_{2.5}$ exposure might affect DNA methylation in fibroblasts, we treated fibroblasts for 72 hours with different doses of $PM_{2.5}$ (0, 3, 30 μg/cm^2) and assayed DNA methylation via the Illumina EPIC DNA methylation array. This array probed 866,091 potential methylation sites with 51,198 probes removed from the analysis due to their proximity to a single nucleotide polymorphism, their known cross-reactivity, or both. This left 811,341 probes for

downstream analysis. Compared to no-$PM_{2.5}$ control, a low (3 ug/cm^2) concentration of $PM_{2.5}$ caused hypermethylation of 138 CpG sites and hypomethylation of 226 CpG sites. Compared to no-$PM_{2.5}$ control, a high (30 ug/cm^2) concentration of $PM_{2.5}$ caused hypermethylation of 141 CpG sites and hypomethylation of 109 CpG sites. Table 4.1 summarizes the results for differential methylation over all probes. Many of these probes corresponded to specific genes; the full list of differentially methylated genes for each comparison is shown in Table 4.2 and Appendix A. Volcano plots of the differentially methylated probes are shown in Figure 4.2B, 4.2D, and 4.2F. DMPs for each comparison were also annotated to a genomic region (i.e. CpG islands, shores, intergenic, etc.) (Figure 4.2B/4.2D/4.2F). These results show that our DMPs trend toward overrepresentation in intergenic regions and underrepresentation in CpG Islands.

We next compared the genes that were differentially methylated in the "Control vs. Low", and "Control vs. High" comparison groups. Nearly the same number of differentially methylated genes were in each group. Interestingly however, the genes that were differentially methylated after low concentration of $PM_{2.5}$ (Figure 4.3A) were mostly different than the genes that were differentially methylated after high concentration $PM_{2.5}$ treatment. Out of the hundreds of genes affected by either low or high concentrations of $PM_{2.5}$ only 15 were affected by both. These data indicate that $PM_{2.5}$ exposure can have significantly different effects on methylation at different concentrations. Furthermore, the biggest differences in methylation patterns were observed when comparing the "Low vs. High" $PM_{2.5}$ treatment groups (Figure 4.3B), which again indicate that the effects of $PM_{2.5}$ on DNA methylation vary depending on concentration.

Gene ontologies enriched among genes differentially methylated by $PM_{2.5}$

To gain further insight into the effect that different concentrations of $PM_{2.5}$ had on DNA methylation following exposure, we performed GO Enrichment analyses on the genes that were

differentially methylated following either low or high concentration $PM_{2.5}$ exposure. If a gene was shown to be differentially methylated in our "Control vs. Low", or "Low vs. High" comparison groups following low concentration exposure specifically, then it was included in our analysis for low concentration $PM_{2.5}$ exposures. Similarly, if a gene was found to be differentially methylated in our "Control vs. High", or "Low vs. High" comparison groups following high concentration exposure, then it was included in our analysis for high concentration $PM_{2.5}$ exposures. Our analysis showed that different concentrations of $PM_{2.5}$ enrich different Go biological processes. Specifically, the Regulation of the Cell Cycle and Response to Cellular Stress were enriched following low concentration exposures. However, in response to high concentration exposures, many processes, including Cell Metabolism, Cell Signaling, Regulation of Neurogenesis, and Regulation of Anatomical Structure Morphogenesis were enriched (Figure 4.3C and Table 4.3).

$PM_{2.5}$ exposure promoted an increase in IL-12 in pulmonary fibroblasts

Because BMP2 is a cytokine that we discovered was robustly upregulated by $PM_{2.5}$, we sought to determine if other cytokines may also be secreted by fibroblasts. We did this by measure secreted cytokines in the supernatant of cells using a Luminex array that samples 41 cytokines. In this array, IL-12 was the only cytokine that was significantly upregulated following low concentration Beijing-$PM_{2.5}$ exposure (Figure 4.4A). There were, however, trends toward increased secretion of several other cytokines (IFNg, MDC, IL-6, VEGF, sCD40L, IL-1RA) in response to various $PM_{2.5}$ exposures, but these effects were not statistically significant (Figure 4.4B).

4.4 Discussion

In this Aim, we assessed the effect that $PM_{2.5}$ exposure had on DNA methylation. We focused on epigenetic changes because we had observed that at least two exposures to $PM_{2.5}$ were required for myofibroblast differentiation, prompting us to hypothesize that epigenetic changes may be occurring that prime the cells for myofibroblast differentiation prior to subsequent exposure. DNA methylation is an important epigenetic mark and regulator of gene expression that has been well-described to be altered by $PM_{2.5}$[78, 80, 81, 82]. Despite being a pilot, exploratory experiment with only three replicates, we did find measurable changes in methylation patterns across the genome after $PM_{2.5}$ exposure. Notably, the effect on methylation was dependent on the concentration of $PM_{2.5}$ the cells were exposed to, with low and high concentrations affecting the methylation of different sets of genes. Furthermore, follow-up GO Enrichment analysis showed enrichment of different sets of GO terms following exposure to different concentrations of $PM_{2.5}$ suggesting that these differences in gene methylation may be functionally significant as GO terms consist of functionally related genes. Notably, several of the differently methylated genes were directly or indirectly related to processes discussed in previous Aims.

The enriched cellular processes in our GO analysis included GO terms, some of which were expected and some of which were novel based on what others have shown or described with $PM_{2.5}$ exposure. $PM_{2.5}$ is known to cause oxidative stress and induced ROS production[73]. Thus, enrichment of the term “Cellular Response to Stress” in response to low concentration $PM_{2.5}$ exposure is not surprising and demonstrates that even low concentration exposures can impact the cell. High concentration exposures enrich a variety of processes, including

"Regulation of Neurogenesis" and "Regulation of Anatomical Structure Morphogenesis". This was notable because BMP2 is also known to regulate these processes[115, 116].

While many genes were differentially methylated following $PM_{2.5}$ exposure, there were a few in particular that were directly relevant to this research. Collagen is a marker of myofibroblast differentiation and many different collagen genes (COL21A1, COL19A1, COL23A1, COL6A1, COLGALT2) were differentially methylated following $PM_{2.5}$ exposure. $PM_{2.5}$ exposure also promoted the methylation of various MAPK (MAP4K3, MAP3K13, MAP4K4) genes. BMP2 signals through MAPK, meaning that methylation of those genes could affect BMP2 signaling. The gene, NFKB1 was hypomethylated following high concentration $PM_{2.5}$ exposure, which was interesting given the role that NF-κB played in the induction of myofibroblast differentiation following repeated $PM_{2.5}$ exposure. While this study does not address the mechanism by which $PM_{2.5}$ causes methylation changes directly, prior work has shown that $PM_{2.5}$ exposure causes upregulation of DNA methyltransferases through ERK pathway activation in T cells[158], while a study in lymphocytes showed that NF-κB activation may lead to cascading changes in DNA methylation[159].

These results raise questions that can be addressed in future studies. We can assess gene expression and protein levels for the collogen genes that were differentially methylated in response to $PM_{2.5}$ exposure. Furthermore, a relatively high number of MAPK genes were also differentially methylated. Given that in Aim 2, we detected increases in BMP2, a cytokine known to signal through MAPK, future studies should attempt to elucidate the effect that $PM_{2.5}$ exposure has on MAPK signaling. While this study that looked at the effects of $PM_{2.5}$ exposure following a single, 72-hour exposure was enlightening, given the results of our Aim 1 study, future methylation studies should also examine the effects of low concentration repeated

exposures. In particular, we would be interested in seeing if the methylation of NFKB1 was affected. To further appreciate the results of any future methylation analysis, we could simultaneously perform an RNA-Seq to assess how methylation affects gene expression. Our sample size was limited in these experiments, and we also observed moderate batch variability, which further limited our statistical power and meant that none of the DMPs passed the false discovery rate (FDR) significance threshold. Given that this was an exploratory, hypothesis generating study, we lowered the significance threshold to $p < 0.005$. Follow-up studies should account for this batch variability and power their study appropriately.

In addition to our methylation studies, we also examined the effect that $PM_{2.5}$ exposure had on the release of cytokines and other secreted analytes. We did this by exposing fibroblasts to $PM_{2.5}$, collecting the supernatant, and analyzing that supernatant with the Luminex immunoassay. We found that one cytokine, IL-12, was significantly upregulated in response to any of our $PM_{2.5}$ exposures. IL-12 has been shown to have a significant role in promoting immune response and the production of interferon-gamma and tumor necrosis factor-alpha[160]. Furthermore, IL-12 has been shown to attenuate bleomycin-induced fibrosis[161], while its subunit, p40, has been shown to have the opposite effect[162]. The increase in only one cytokine was unexpected as other studies have found that $PM_{2.5}$ had significant effects on cytokine secretion in fibroblasts[66, 67, 163]. One potential reason for this was that we only assayed for 41 proteins out of all possible secreted mediators. Given the ability to cast a wider net we may have uncovered more significant effects. Another factor that may have contributed to this was the high variability across experiments. While some variability is unavoidable due to the heterogeneous nature of $PM_{2.5}$, future experiments must take this into account. Previously, we overcame this inherent variability by increasing the sample size. In our present experiment, there were trends toward

increases in some of the other cytokines in our panel, specifically IFNg, MDC, IL-6, VEGF, sCD40L, and IL-1RA. By increasing the sample size and subsequently the statistical power, future Luminex assays will be able to determine if these increases are genuine or statistical noise. Furthermore, the results of these future Luminex experiments could be compared with the results of other analyses, such as future DNA methylation arrays, to provide a more comprehensive and complete look into the effects of $PM_{2.5}$ exposure on important aspects of fibroblast biology.

One of the goals we had at the outset of this Aim was to generate new hypotheses and inform future research directions. Our main take away in this regard, is that different concentrations of $PM_{2.5}$ can have different effects. We have now seen significant differences in methylation profiles following low and high concentrations of $PM_{2.5}$ exposure. Whether these differences in DNA methylation affect gene expression or other fibroblast functions are unknown, but do provide intriguing hypotheses for future exploration. Taken together, these results suggest that future $PM_{2.5}$ studies should examine the effects of a wide concentration range in order to fully appreciate the impact of the exposure they are studying.

4.5 Tables

Table 4.1: Summary of methylation results

Comparison	Contrast	Hyper Meth.	Hypo Meth.	No DM	Filter By	Sig. Thresh.	Delta Beta
Control v Low	Low - Control	138	226	810,977	pval	0.005	5
Control v High	High - Control	141	109	811,091	pval	0.005	5
Low v High	High - Low	1,170	674	809,497	pval	0.005	5

Table 4.2: Differentially methylated genes

Section I: Low Exposure (relative to control)			Section II: Overlap		Section III: High Exposure (relative to control)	
Hyper-	Hypo-		Hyper-	Hypo-	Hyper-	Hypo-
ADIPOR1	ADCY5	PKD1L3	SHE	TRIM34	ABCA8	ACSF3
AGPAT3	ADRA1A	POLK	ABCB1	HBG2	ABCG2	ADGRL2
ALDH8A1	AGBL4	PPP2R2B	ODF2L	FSTL5	ABHD6	ALOX5AP
ANKRD44	AKAP13	PRKCZ	ZIC2	URI1	ACAP2	ATP6V1G1
AP5S1	ANO3	PRSS27	SLAIN1	TRIM6-TRIM34	AQP11	AXIN2
APOL2	ARFGEF2	PTPRG	OXR1	C1QTNF1	ARAP2	C10orf90
APOL4	ARID5B	PTPRS		LCTL	ATP11AUN	CA1
APOPT1	ARL9	Pvt1		c9orf3	ATXN1	CA13
ASH2L	ARPP21	RAB37		WDFY2	BET1	CCDC113
ATCAY	ATP1A1	RAPGEF1			BUB1B	CCDC6
AUNIP	BRSK2	RPL8			C9orf78	CCDC85A
BAG5	BTNL3	SBSPON			CCDC30	CCRL2
BEND6	CASC1	SCART1			CELF1	CEP135
BTBD16	CCDC158	SCD5			CEP85L	CLEC3A
C2CD2L	CCDC160	SGSM1			CLCN3	CLINT1
CDH18	CCDC60	SLC24A4			COL21A1	CNPY1
CDH4	CCNY	SNAPIN			CUL1	CWC25
CHCHD3	CD300LF	SNORD115-7			DAB1	ERG
CHRD	CDH18	SNORD115-8			DOK6	ETV1
CLEC16A	CDK17	SNORD115-9			EPS15	EXT1
COL19A1	CDK19	SRPX2			ERBIN	EYS
CST3	CNOT6	STIL			ERC2	FBXO15
DEXI	COMMD7	SYNRG			ESR1	FSTL4
DHFR	CPA5	TAL1			ETAA1	FUT10

DNAH7	CRYZL1	TBX5			EYA4	GALNT14
DPAGT1	CTBP2	THBS2			FAR2	GAREM2
DTNB	CYYR1	THRAP3			FARS2	GRM3
EHBP1	DCAF10	TKTL2			FCER1A	H3F3A
ERCC6L2	DDAH2	TMEM132D			FGD6	H3F3AP4
EXOC5	DLX6-AS1	TMEM168			FLJ37505	ICA1
FAM129B	DMKN	TNRC6C			FNDC1	JARID2
FAM170B	DONSON	TNS3			FUT10	KIAA1217
FAM170B-AS1	EBF1	TOR2A			GCM2	KIF18A
FAM20C	EIF4G3	TPK1			GMCL1	KRTAP20-4
FKBP15	EMCN	TRIM26			GOLGA3	KRTAP6-1
GALNT13	EPB41	TRPA1			INPP5A	KTN1
GALNT5	EPB41L3	TUSC3			IQSEC2	LBR
GLRA3	ESRRG	TYW1			KIAA0586	LHFPL1
GPHN	EVA1A	UBE2D3			KIDINS220	LINC00689
GYG1	EXOC4	UBE3D			KLF7	METTL15
Hist1h3i	FAM161A	UQCRBP1			LINC00968	METTL25
IDO1	FBXL2	VEZT			LMO4	MIR100HG
IL15RA	FBXO31	VPS41			LOC100507564	MIR216B
IRAK4	FER1L6	WDFY3			LRRC20	MIR548AP
KCNAB1	FER1L6-AS1	WDR45B			LUM	MTFMT
KCNAB1-AS2	FGD6	YTHDC2			LVRN	MYO6
KLC1	FHIT	ZFHX3			MAP10	Mir125b1
KNTC1	FLI1	ZNF517			MAP4K3	NFKB1
LAMC2	FNBP1	ZNF559			MEP1A	NMD3
LINC00332	FTO	ZNF559-ZNF177			MIR181A1	OR1D2
LINC00476	GABRA4	ZNF608			MIR181A1HG	OR5P3
LINC01091	GADL1	exoc2			MS4A2	PAX6
LINC01623	GFPT2	hla-dqa1			MSH2	PCAT19
LOC728554	GGACT	josd2			MTA2	PDZRN3
LRP5	GJB7	kcnk15			MYCT1	PIEZO2
MACROD2	GPC5	mir124-2			MYOM2	PIP4K2B
MC2R	GRID1	ptdss2			Mir181b1	PRKAR1B
MCF2L2	HCAR1	tmem170b			NCLN	RBMX
METTL25	HDAC9				NEIL2	REPS1
MFSD11	HNRNPC				NEK7	RGS17
MIR636	HOTTIP				NOX4	RPS7
MKL2	HOXA13				NPHP3	SELP
MNAT1	HUS1B				OR2AT4	SERBP1
MS4A5	IFITM10				PAK6	SLC16A4
MSH3	IGSF21				PCDHA1	SLC22A2

MTRNR2L2	IL18RAP			PCDHA10	SLC25A3
MYO15A	ITPKB			PCDHA2	SLC6A18
NEDD4L	ITSN1			PCDHA3	SLC6A19
NEMF	JKAMP			PCDHA4	SLC7A2
NKAIN2	JPH3			PCDHA5	SNORD61
OR10G4	KBTBD12			PCDHA6	SV2B
OR4C6	KIRREL2			PCDHA7	TANGO6
OR51E2	KRTAP13-2			PCDHA8	TAPBP
OR8A1	Krtap4-8			PCDHA9	TIMM21
PARD3	LAMP1			PDXP	TMTC1
PCDH11X	LAT2			PHF14	TRIM2
PDE1A	LDB2			PLN	TRPM4
PDGFRA	LINC01420			PRR7	TTLL7
PDIA6	LNX1			PRR7-AS1	WDR66
PKNOX2	LNX1-AS2			PTCHD4	ZNF704
PLCZ1	LOC374443			PTN	epha6
PXN	LPP			RALA	il6st
RAB30	LRBA			RAPGEF1	map4k4
RAB30-AS1	LRRC6			RPS6KA5	
RBMS3	MAGED2			SLC25A4	
REV1	MAGI1			SNX2	
RGS14	MAML2			SNX7	
ROR2	MANEA			SORBS2	
RPS18	MAP1LC3B			STXBP5	
RSL1D1	MAP3K7			STXBP5-AS1	
RSRC2	MED12L			SUPT20H	
SETDB2	METTL9			SYCP2L	
SFPQ	MIR124-2HG			SYNJ1	
SKAP1	MIR548AX			TAPT1	
SLC13A1	MOB2			TCEB1	
SLC31A1	MOV10			TCF25	
SLC38A10	MTUS1			TMEM135	
SMAD2	NEU1			TMEM161B	
SORD	NEUROG1			TNFSF12	
SRSF2	NFATC2			TNFSF12-TNFSF13	
TAOK1	NOP58			UBASH3A	
TARS	NPHS1			UBQLN2	
TBX3	NYAP2			UNC5D	
TGIF2-C20orf24	OPCML			USP20	
THPO	OR13C3			VSIG4	
THSD7B	OR13C8			WWC2	

TIAL1	OR51A2			ZBTB46	
TMEM108	PAK3			ZC3H12C	
TMEM97	PARVB			ZCCHC7	
VPS52	PCAT4			ZNF423	
WDR66	PDGFRA			ZNF578	
ZBTB38	PHACTR3			c6orf106	
ZNF117	PHF21B			fktn	
hist1h4l	PIK3R1			magoh	
	PINK1			sh3bp1	

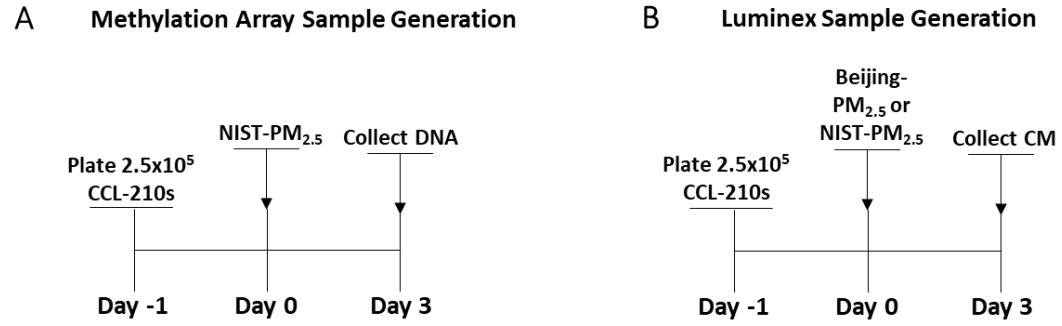
Table 4.3: Results of GO Enrichment analysis

	GO Terms	Number of Enriched Genes	Expected Number of Enriched Genes	Fold Enrichment	FDR
Low Exposure	Regulation of Cell Cycle	29	11	2.64	0.0515
	Cellular Response to Stress	91	54.43	1.67	0.0533
High Exposure	Cell Adhesion	30	8.72	3.44	0.000134
	Regulation of Neurogenesis	28	11.69	2.39	0.0452
	Neuron Projection	48	23.12	2.08	0.00786
	Regulation of Anatomical Structure Morphogenesis	80	47.39	1.69	0.0144
	Regulation of Cellular Organization	95	58.41	1.63	0.00876
	Cell Communication	221	169.22	1.31	0.0303
	Cell Signaling	221	170.01	1.3	0.035
	Cellular Metabolic Process	431	366.89	1.17	0.0388

Legend – Differentially methylated genes were divided into 2 groups for GO Enrichment analysis. The first group (Low Exposure) consisted of genes that were hyper or hypo methylated in the Low Exposure (Relative to Control) comparison group as well as genes that were hypermethylated following low concentration exposures in the Low vs. High (head-to-head comparison) comparison group. The second group (High Exposure) consisted of genes that were hyper or hypo methylated in the High Exposure (Relative to Control) comparison group as well as genes that were hypermethylated following high concentration exposures in the Low vs. High (head-to-head comparison) comparison group.

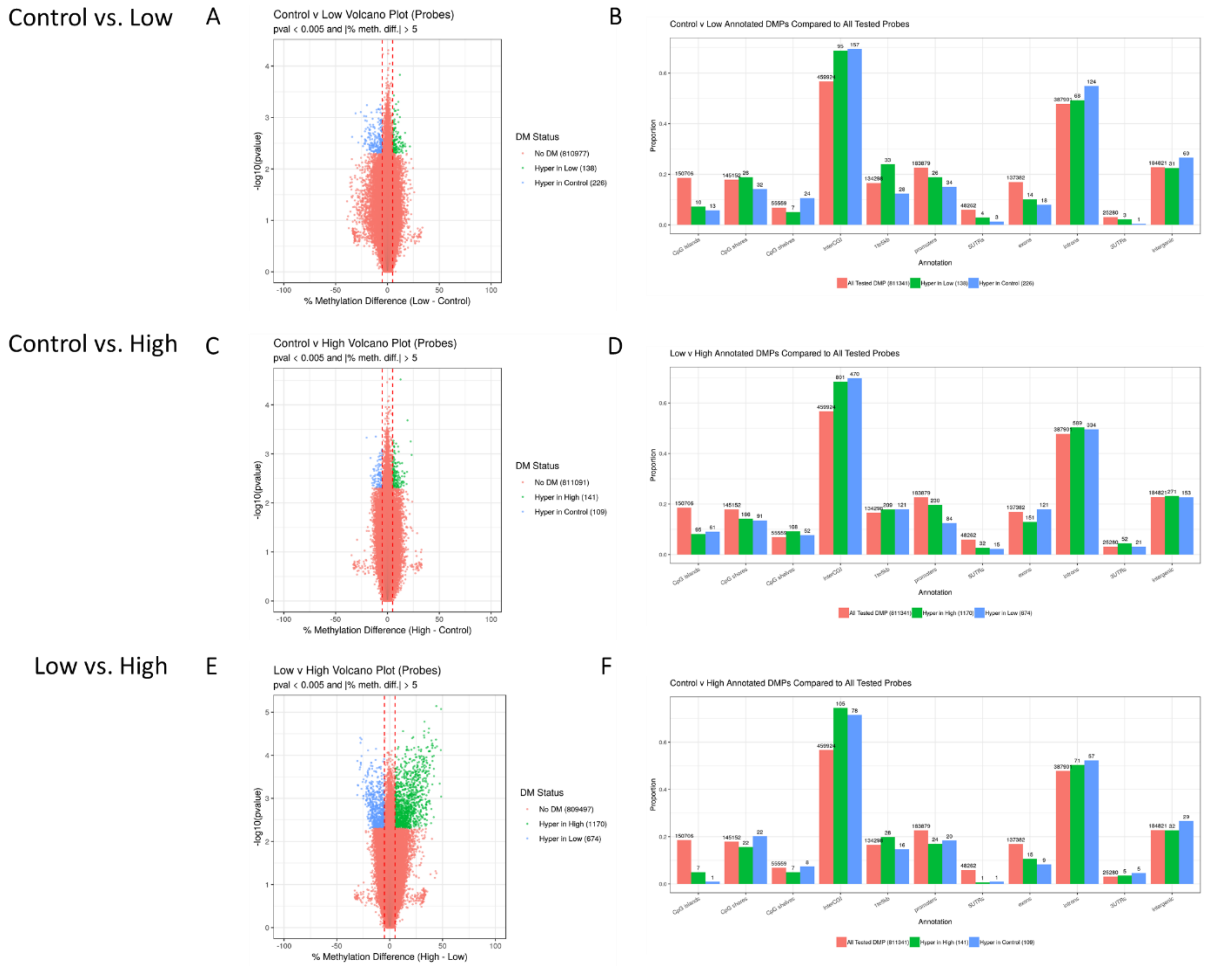
4.6 Figures

Figure 4.1: Methods for sample generation



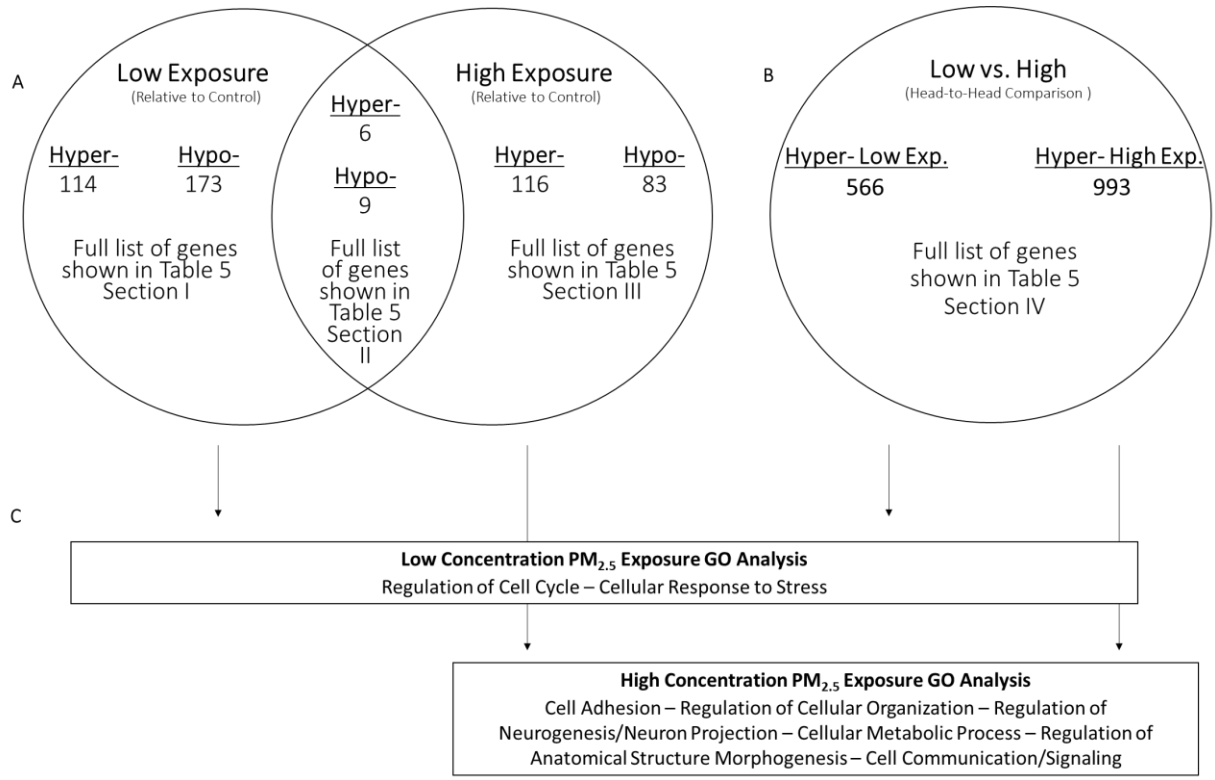
Legend – Wire diagrams for sample generation for the Methylation studies (A) and the Luminex studies (B). Abbreviations: CM – Conditioned Medium.

Figure 4.2: $PM_{2.5}$ exposure promotes differential methylation in pulmonary fibroblasts



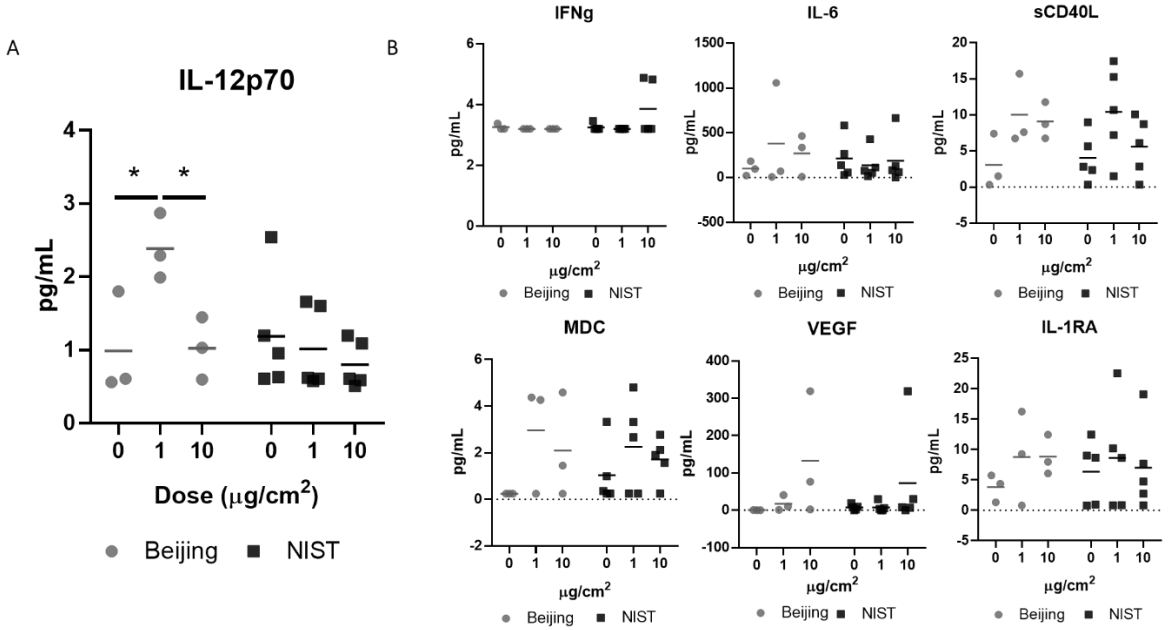
Legend – Changes in DNA methylation following a 3 day single exposure to NIST-$PM_{2.5}$. Volcano Plots for the "Control vs. Low", "Control vs. High", and "Low vs. High" comparison groups are shown in A, C, and E, respectively. DMPs for the "Control vs. Low", "Control vs. High", and "Low vs. High" comparison groups were also annotated to a genomic region (B, D, and F, respectively).

Figure 4.3: Gene Enrichment analysis of genes differentially methylated by $PM_{2.5}$



Legend – Comparison of the genes differentially methylated by low concentration and high concentration exposures to NIST-$PM_{2.5}$, as well as the results of the subsequent GO Enrichment Analysis. A) Comparison of the genes differentially methylated in the "Control vs. Low" comparison group and the "Control vs. High" comparison group. B) Direct comparison of gene methylation in fibroblasts exposed to low and high concentration of NIST-$PM_{2.5}$ C) Summary of the results of the Go Enrichment analysis.

Figure 4.4: $PM_{2.5}$ exposure promoted an increase in IL-12 in pulmonary fibroblasts



Legend – The effect of a 3 day single $PM_{2.5}$ exposure on cytokine levels in pulmonary fibroblasts, assessed via Luminex assay A) IL12-p70 levels following 3 day single exposure to $PM_{2.5}$. B) Levels of 6 other select cytokines following 3 day single exposure to $PM_{2.5}$. The remaining analytes were detected but showed little to no change, significant or otherwise. *$p<0.05$ relative to control.

Chapter 5 The Effect of $PM_{2.5}$ Exposure on Bleomycin-induced Fibrosis

5.1 Introduction

Our work so far has focused on the effects of $PM_{2.5}$ exposure on fibroblast biology *in vitro*. However, whether these effects contribute to fibrosis *in vivo* is uncertain. Since fibroblasts play a critical role in the development of fibrotic lung diseases, their activation by $PM_{2.5}$ could contribute to the development of pulmonary fibrosis. Previously, other investigators have used animal models to explore the impact of $PM_{2.5}$ exposure on the development of pulmonary fibrosis. They found that $PM_{2.5}$ exposure *in vivo* leads an increase in a variety of fibrosis related endpoints, including Col1a1 deposition, αSMA expression, increases in epithelial-to-mesenchymal transition, and worsened lung histology[46, 164 - 170]. Interestingly, a wide a variety of exposure protocols were utilized in the literature. These included exposure concentrations that ranged from 2.5 ug/mouse to 3 mg/mouse for discrete exposure methods such as internasal, oropharyngeal, and intratracheal exposures, and from 59.77 ug/m^3 to 300 ug/m^3 for more continuous, inhalation exposures. Additionally, the number and duration of the exposures varied from a single instance to weeks or even months of repeated exposures.

Liu *et al.* found that mice exposed to inhaled PM alone for 12 weeks developed severe lung injury and fibrosis[153]. Another study showed that mice given PM intranasally for 4 weeks developed lung inflammation and fibrosis, as confirmed by micro-CT imaging and histology[46]. These mice exhibited elevated levels of TGF-β, αSMA, and Collagen Type 1 (COL1) in their lungs. Both of these studies demonstrate that chronic, repeated exposure to PM can induce fibrotic changes in mice, but the mechanisms of fibrosis are uncertain, and the minimum

Exposure necessary for triggering fibrosis is unknown. Xu *et al.* found that rats exposed to repeated doses of $PM_{2.5}$ concurrently with bleomycin, a common chemotherapeutic agent used to induce lung fibrosis, developed increased collagen and αSMA in the lungs and worse overall histologic fibrosis[171]. The concurrent treatment with $PM_{2.5}$ during bleomycin administration however may simply increase the overall oxidative stress of bleomycin injury and does not indicate whether early $PM_{2.5}$ exposure might affect later susceptibility to fibrosis. Our in vitro studies in normal, quiescent fibroblasts demonstrated that low concentrations of $PM_{2.5}$, repeated just once, was sufficient to cause these cells to differentiate into myofibroblasts. This led us to hypothesize that pre-exposure of mice to $PM_{2.5}$ – potentially in repeated fashion – may increase susceptibility of mice to further fibrosis from bleomycin.

Although the aforementioned studies suggest $PM_{2.5}$ may induce fibrosis *in vivo*, the mechanisms for this are unclear. In the Liu *et al*. study, they performed proteomics and gene ontology analysis of the lungs of mice repeatedly exposed to $PM_{2.5}$ for 12 weeks. They found elevations in extracellular matrix (ECM)–receptor interactions, and enrichment of the GO terms for the phagosome, and phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt) signaling pathways that coincided with the PM-induced effects on lung fibrosis[172]. Because our *in vitro* data suggests that only two $PM_{2.5}$ exposures are sufficient to induce myofibroblast differentiation, we sought here, to determine whether pre-exposure to $PM_{2.5,}$ which may have the potential to activate fibroblasts, may increase susceptibility of animals to subsequent bleomycin-induced fibrosis. Given that we had also determined that $PM_{2.5}$ effects on fibroblasts occurred through an NF-κB dependent manner, we also sought to determine the mechanistic contributions of NF-κB in this process.

5.2 Methods and Materials

Acquisition of Mice

Wild type 8-10 week-old male and female C57Bl/6 mice were purchased from Charles River Laboratories. In the first experimental flight, 20 male mice were used and in the second flight 45 female mice were used.

$PM_{2.5}$ Collection and Preparation

For our mouse exposure experiments we utilized $PM_{2.5}$ from the National Institute of Standards and Technology (i.e. the NIST-$PM_{2.5}$ from prior Chapters). As previously mentioned, this $PM_{2.5}$ was collected from Washington, D.C. in 1976 and is well-characterized and studied by many investigators over many years. The $PM_{2.5}$ is shipped as a solid and was solubilized in double distilled water by sonication (VWR, model no. 97043–968, VWR International, Radnor, Pennsylvania, USA). Following solubilization, aliquots were stored at –80 °C until use.

The dose of $PM_{2.5}$ utilized (100 µg per mouse) was calculated based on the approximate minute ventilation of a mouse (~57 mL per minute), and the atmospheric concentration of $PM_{2.5}$ in areas that experience high levels of air pollution such as Southeast Asia, India, and Northeast China[173, 174]. Additionally, this dose is similar to the doses used by other *in vivo* studies that looked at the effects of $PM_{2.5}$ exposure on lung fibrosis. The mice in the control group received saline. $PM_{2.5}$ was concentrated in a volume of 50 µl.

Mouse Exposure Protocol

To assess the effect of $PM_{2.5}$ exposure on the development of pulmonary fibrosis, mice were oropharyngeally exposed to $PM_{2.5}$ every 3 days up to 2 times (day -6, -3) prior to treatment with either bleomycin (1.1 U/kg, Sigma Aldrich, St. Louis, MO, USA) or saline (day 0) (Fig 5.1A). We intentionally used a lower dose of bleomycin than what is typically used by other

investigators because we hypothesized that $PM_{2.5}$ would increase the susceptibility of animals to fibrosis from lower concentrations of bleomycin. We sought to ensure that bleomycin treatment alone would not cause such severe fibrosis that we would be unable to assess the impact of $PM_{2.5}$ exposure. The weight of the mice was monitored throughout the experiment with weights taken on days -7, -1, 7, 14, and 21. On day 21, the mice were euthanized, and their lungs collected post-mortem for histology and hydroxyproline measurements. Hydroxyproline is measured by homogenizing the lungs and performing an absorbance-based assay on the homogenized sample. These results are compared to results from a standard curve of known hydroxyproline concentration.

Statistical Analysis

The significance of the hydroxyproline assay data and body weight data was assessed by ANOVA. Histological sections were assessed qualitatively by visually comparing experimental conditions.

5.3 Results

Preliminary experiments suggest pre-treatment with $PM_{2.5}$ increased susceptibility to bleomycin-induced pulmonary fibrosis.

Male mice treated with bleomycin had higher average levels of hydroxyproline although the effect was mild and not significant. This was likely due to the low dose of bleomycin used, as bleomycin treatment normally promotes a larger increase in hydroxyproline. Mice exposed to $PM_{2.5}$ exhibited a trend towards increased hydroxyproline after bleomycin treatment compared to bleomycin-treated mice that were not exposed to $PM_{2.5}$, though the difference was not statistically significant (Figure 5.1B). Qualitative assessment of lung histology also showed that

lung fibrosis was worse in mice that were pre-exposed to$PM_{2.5}$ prior to bleomycin treatment compared to mice that received saline prior to bleomycin treatment (Figure 5.1C).

A repeat experiment in female mice did not confirm an increase in fibrosis associated with pre-treatment with $PM_{2.5}$

Because the results above were preliminary and not statistically significant (likely due to being underpowered), we tried to repeat the above experiment in female mice (Figure 5.2A). In this follow-up experiment, mice subjected to single or repeated $PM_{2.5}$ exposure did not exhibit lung fibrosis as measured by hydroxyproline (Figure 5.2B). Interestingly, mice exposed to $PM_{2.5}$ prior to bleomycin installation did not have worse lung fibrosis than control mice that were treated with PBS and then instilled with bleomycin. Notably, mice that were exposed to $PM_{2.5}$ repeatedly prior to bleomycin installation actually had slightly lower hydroxyproline content in their lungs compared to mice that received bleomycin and either PBS or a single $PM_{2.5}$ exposure. In addition to the hydroxyproline data, a qualitative assessment of histological sections taken from the mice support the conclusion that $PM_{2.5}$ exposure has minimal effect on bleomycin induced pulmonary fibrosis in this follow-up experiment (data not shown).

When examining the weight of mice during the exposure, we found that exposure to bleomycin alone, led to a small drop in weight by day 7, as commonly observed and expected (Figure 5.2C). However, when mice were exposed to $PM_{2.5}$ in conjunction with bleomycin, there was minimal change in weight. This suggests that pre-treatment with $PM_{2.5}$ may prime mice and lead to compensatory protection from bleomycin-induced injury early on.

5.4 Discussion

In this Aim, we set out to determine whether $PM_{2.5}$ increases susceptibility to pulmonary fibrosis as we had observed in fibroblast experiments *in vitro*, with the goal of using this model

to assess the significance of repeated $PM_{2.5}$ exposures and NF-κB signaling. Unfortunately, despite promising data from our initial experiment (Figure 5.1), where we showed a trend towards worse fibrosis when $PM_{2.5}$ exposure preceded bleomycin treatment, we were unable to replicate the effect with a larger sample size (Figure 5.2).

Interestingly, we did not observe a consistent increase in susceptibility to bleomycin-induced fibrosis when mice were pre-treated with $PM_{2.5}$. This may be due to the fact that the sex of the mice was different in each experimental flight. In the first flight, we utilized male mice and saw trends toward worse bleomycin induced fibrosis following $PM_{2.5}$ exposure that we were not able to replicate in female mice. Prior literature has shown that both male mice and male humans are more susceptible to pulmonary fibrosis[175, 176]. Follow-up studies with both male and female mice in the same flight will be able to shed further light on the possibility of sex playing a significant role in the effects of $PM_{2.5}$ exposure on bleomycin induced fibrosis.

Another reason why we may not have seen a consistent increase in bleomycin induced fibrosis following pre-treatment with $PM_{2.5}$ was the concentration of $PM_{2.5}$ that was used in the pre-treatment exposures. Our *in vitro* data showed that only a narrow range of concentrations promoted myofibroblast differentiation, so it is possible that only a narrow range of concentrations promote fibrosis consistently in this model. If we assessed a wider range of concentrations, we may find one that promotes susceptibility to bleomycin-induced fibrosis more consistently.

Additionally, we exposed mice to $PM_{2.5}$ prior to stimulation with bleomycin. In an unstimulated lung, there are not many fibroblasts, so it is possible that the $PM_{2.5}$ was unable to have consistent profibrotic effects in an unstimulated lung because there were an insufficient number of fibroblasts for $PM_{2.5}$ to act on. We could test this theory in follow-up experiments by

giving bleomycin at the same time as $PM_{2.5}$ or adding $PM_{2.5}$ exposures after bleomycin treatment, so that bleomycin stimulation could promote proliferation of fibroblasts, giving the $PM_{2.5}$ more fibroblasts to act on when it enters the lung.

Another reason for our lack of consistent results is that $PM_{2.5}$ exposure may actually have protective effects in certain contexts. In our larger experimental flight, we found that female mice repeatedly exposed to $PM_{2.5}$ prior to bleomycin treatment had slightly less hydroxyproline than mice that got bleomycin alone although the effect was not significant. A similar phenomenon was also seen in the mouse weight data, where mice that were pretreated with $PM_{2.5}$ were protected from weight loss following bleomycin installation. There are also other studies that suggest that $PM_{2.5}$ exposure can promote changes in antioxidant defense that confer protection from subsequent environmental challenges[153, 177].

Others that have shown that $PM_{2.5}$ exposure promoted pulmonary fibrosis *in vivo* often utilized exposure protocols with longer exposure periods and more exposures. Specifically, studies using discrete exposures (internasal, oropharyngeal, or intratracheal) had success with up to 28 exposures spread over as many days[46]. Studies that utilized inhalation exposures tended to be even longer with more continuous exposures[165, 167]. Follow-up studies that compare the effect of different exposures protocols could help determine the optimal method for achieving consistent results.

Finally, variability within the study, and a lack of statistical power may have contributed to our inability to consistently show an increase in susceptibility to bleomycin-induced fibrosis in mice following $PM_{2.5}$ exposure. While there were no significant or obvious technical issues that would explain the difference in the results of the pilot and main experiment, there were challenging aspects of this exposure protocol that may have contributed to experimental

variability. These technical challenges include the technique of oropharyngeal exposure, which is performed on lightly anesthetized, often uncooperative mice, as well as pipetting, mixing and aliquoting $PM_{2.5}$, an inherently difficult task due to the compound's resistance to homogenization. These challenges can increase variability, which makes detecting significant changes consistently more difficult.

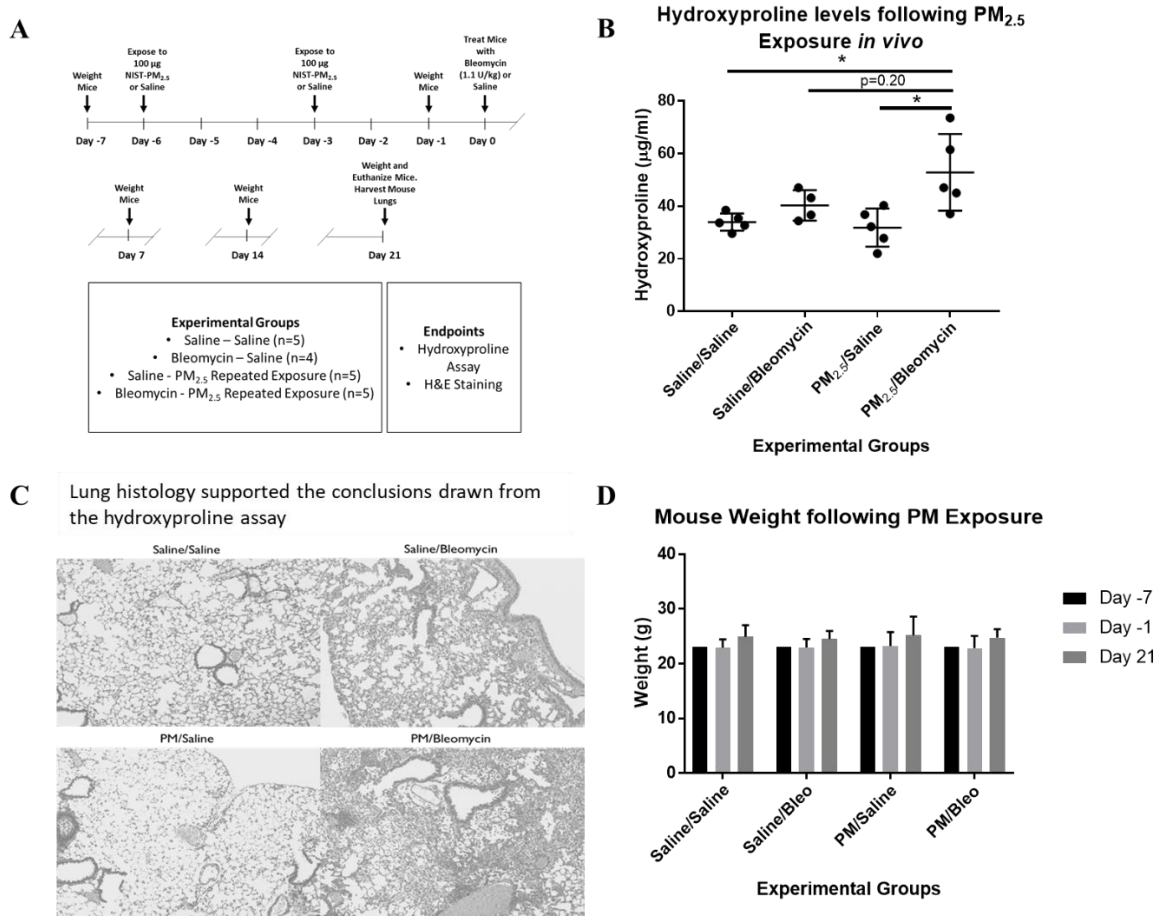
Regardless of the reason for the disparate results between our two flights of mouse experiments, it is clear that additional experimental flights are necessary. To maximize our chance of recreating the results of the first flight, we would ideally utilize male mice in this follow-up study. Alternatively, investigations using both sexes may be able to determine why male mice were more susceptible to fibrosis in our model in the first place. Given additional experimental flights with male mice, we would expect that pretreatment with $PM_{2.5}$ would result in worse bleomycin-induced fibrosis. The results of our Aim 1 experiments show that NF-κB signaling plays a significant role in how $PM_{2.5}$ affect myofibroblast differentiation. To assess NF-κB's effect *in vivo*, we would utilize the same NF-κB inhibitor that we used to block the increase in myofibroblast differentiation following repeated low concentration $PM_{2.5}$ exposure. We expect that mice who are exposed to $PM_{2.5}$ but not treated with a NF-κB inhibitor will show worse fibrosis than conditions that did receive an NF-κB inhibitor. These findings would be significant as they would provide *in vivo* data to support one of our main conclusions in Aim 1; that activation of NF-κB is necessary for $PM_{2.5}$ exposure to have profibrotic effects.

While further work could provide compelling data concerning $PM_{2.5}$ exposure and fibrogenesis, for the moment, we are left with our current *in vivo* data. Despite promising preliminary results, where repeated $PM_{2.5}$ exposure preceding bleomycin installation caused a non-significant increase in fibrosis as measured by collagen content in the lungs, follow-up

experiments with larger sample sizes were unable to replicate these results. Based on the results of this experiment, any follow-up studies should be aware of the effect that the sex of the mice may have on the study. Furthermore, care should be taken to utilize enough mice to achieve statistical significance, as $PM_{2.5}$ exposures often have more inherent variability than more homogeneous treatments.

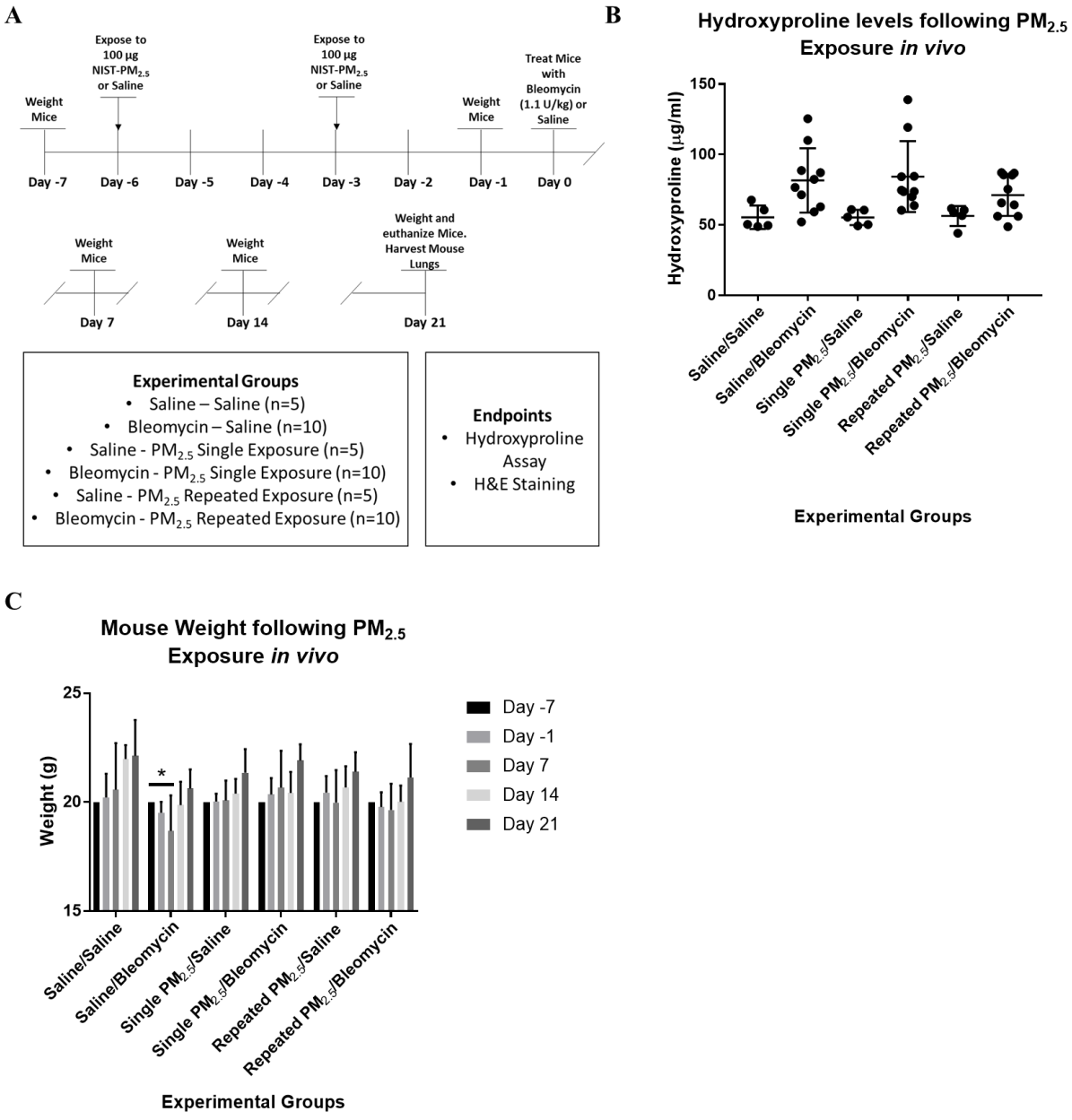
5.6 Figures

Figure 5.1: Preliminary experiments suggest pre-treatment with $PM_{2.5}$ increased susceptibility to bleomycin-induced pulmonary fibrosis.



Legend – Procedure and data from our first mouse experiment utilizing male mice. A) Wire Diagram of the *in vivo* exposure protocol, as well as experimental groups and endpoints. B) Hydroxyproline levels following $PM_{2.5}$ exposure *in vivo* C) Lung histology following $PM_{2.5}$ exposure *in vivo* D) Body weight following $PM_{2.5}$ exposure *in vivo*. *$p<0.05$ relative to control.

Figure 5.2: Repeat experiments in female mice did not confirm an increase in fibrosis associated with pre-treatment with $PM_{2.5}$



Legend – Procedure and data from our second mouse experiment utilizing female mice. A) Wire Diagram of the *in vivo* exposure protocol, as well as experimental groups and endpoints. B) Hydroxyproline levels following $PM_{2.5}$ exposure *in vivo* C) Body weight following $PM_{2.5}$ exposure *in vivo*. *$p<0.05$ relative to control.

Chapter 6 Public Health Implications and Future Plans

6.1 Overview

At the beginning of this dissertation, we set out to investigate the effects of $PM_{2.5}$ on the activation of fibroblasts and ability to cause fibroblasts to differentiate into myofibroblasts, an important process in the onset of fibrotic diseases such as IPF. Epidemiological research has shown that $PM_{2.5}$ exposure can contribute to sub-clinical interstitial lung disease, a potential precursor lesion of pulmonary fibrosis[51]. Additionally, prior work has tentatively shown that $PM_{2.5}$ exposure causes myofibroblast differentiation *in vitro*[46]. Our study sought to build on this prior work by further characterizing the effect of $PM_{2.5}$ exposure on fibroblasts. Specifically, we examined the actions of different sources of $PM_{2.5}$, as well as how different concentrations, frequency and duration of exposure alters fibroblast phenotype. Finally, we sought to undercover the mechanisms driving these changes, focusing on the roles that NF-κB and BMP2 had in causing myofibroblast differentiation following $PM_{2.5}$ exposure. We utilized a global DNA methylation array and Luminex assay to gain further insights into other actions and mechanisms by which $PM_{2.5}$ alters fibroblast phenotype. Finally, we attempted to translate our *in vitro* data to a mouse model of fibrosis.

We found that $PM_{2.5}$ exposure caused an increase in myofibroblast differentiation. Notably, this increase occurred in response to repeated exposures to low concentrations of $PM_{2.5}$ rather than a single exposure to a higher concentration, as was shown in previously published work[46]. Mechanistically, NF-κB activation was necessary for $PM_{2.5}$ exposure to have these

effects, while the role of BMP2 was more nuanced. Furthermore, broader studies looking at DNA methylation and secreted cytokines found that there were striking differences in the effects of different $PM_{2.5}$ exposure levels. These findings were significant as they show that $PM_{2.5}$ itself is capable of promoting myofibroblast differentiation, even without the presence of other pro-fibrotic mediators, at low concentrations that are rarely tested or studied. Furthermore, $PM_{2.5}$ has extensive actions on fibroblasts – including affecting BMP2, other cytokines, and DNA methylation, that have never been previously appreciated, and these changes in fibroblast have the potential to ultimately affect the development of lung fibrosis and response to lung injury.

6.2 Aim 1 Summary and Future Plans

In the first Aim, we sought to determine whether $PM_{2.5}$ affects myofibroblast differentiation, and we found that it did. Surprisingly though, the optimal concentration in which $PM_{2.5}$ promoted myofibroblast differentiation was much lower than what any other group has previously shown. Previously, a single exposure to a high concentration of $PM_{2.5}$ was shown to be necessary for inducing myofibroblast differentiation[46]. We however, found that this had no effect on αSMA and collagen production; and may even suppress myofibroblast differentiation. Instead, repeated exposure to low concentrations (0.01 – 1.0 $\mu g/cm^2$) of $PM_{2.5}$ induced increases in αSMA and collagen. NF-κB activation was shown to be necessary for this increase to occur. These results are significant as they demonstrate that small changes in $PM_{2.5}$ levels can have a large impact on fibroblast activation. Given the significant role of fibroblasts in IPF, this in turn implies that small changes in $PM_{2.5}$ exposure can significantly impact risk of developing pulmonary fibrosis.

A potential area of future study is uncovering what it is about the repeated exposure protocol that makes it able to activate fibroblasts when the single exposure protocol failed.

Specifically, follow-up studies that interrogate the changes that occur following a single low concentration $PM_{2.5}$ exposure would be appropriate based on our observations here. We hypothesize that changes following the first exposure to $PM_{2.5}$ renders fibroblast more susceptible to activation and myofibroblast differentiation following subsequent exposures. As we explore in Aim 3, these changes may be epigenetic in nature.

6.3 Aim 2 Summary and Future Plans

While NF-κB is necessary for $PM_{2.5}$-induced myofibroblast differentiation, the downstream factor(s) that mediate the actions of NF-κB are unknown. Here, we investigated whether BMP2 is one such factor. Previous studies have shown that NF-κB is a transcription factor that upregulates BMP2[30]. Our initial experiments showed that treatment with exogenous BMP2 robustly increased myofibroblast differentiation, supporting the idea that $PM_{2.5}$ may be inducing myofibroblast differentiation through upregulation of BMP2. Although we did indeed observe that $PM_{2.5}$ upregulated BMP2 expression in a concentration-dependent manner, the concentration of $PM_{2.5}$ at which BMP2 was elevated was different then the concentration at which myofibroblast differentiation was induced. Moreover, silencing BMP2, or blocking its effect with the inhibitory protein Noggin, did not inhibit myofibroblast differentiation following $PM_{2.5}$ exposure. In fact, blocking BMP2 signaling led to an increase in αSMA at baseline, even without $PM_{2.5}$ treatment. These results indicate that BMP2 may promote myofibroblast differentiation when given exogenously to fibroblasts, but silencing or inhibiting endogenous generation of BMP2 does not prevent $PM_{2.5}$-induced myofibroblast differentiation and suggests that endogenously produced BMP2 may even inhibit myofibroblast differentiation at high concentrations of $PM_{2.5}$ exposure.

Future experiments should attempt to determine the downstream signaling pathway(s) that mediate this dichotomous relationship between BMP2 and myofibroblast differentiation. This is relevant as BMP2 has been shown to signal through two main pathways, the canonical SMAD pathway and the non-canonical MAPK pathway. Given the divergent results of the exogenous BMP2 experiments versus the BMP2 siRNA/noggin experiments, it may be that different conditions result in signaling through distinct pathways. Follow-up experiments testing this hypothesis would provide additional insights into how BMP2 might affect myofibroblast differentiation, and fibrosis, in different contexts.

6.4 Aim 3 Summary and Future Plans

In the third Aim, we attempted to assess the impact of $PM_{2.5}$ exposure on two additional biologic mechanisms, DNA methylation and cytokine secretion. We found that $PM_{2.5}$ exposure caused significant changes in DNA methylation patterns. The most notable aspect of the DNA methylation data was how exposure to different concentrations of $PM_{2.5}$ led to methylation changes in completely different sets of genes. Further analysis using the GO enrichment tool revealed that different GO terms were enriched following exposure to different concentrations of $PM_{2.5}$. This suggests that the effects of $PM_{2.5}$ exposure on DNA methylation are concentration dependent. Future studies can build on this investigation by examining a wider range of $PM_{2.5}$ concentrations. Assessing the effects of repeated exposure to low concentrations of $PM_{2.5}$ on DNA methylation levels is of particular interest given the ability of that exposure to induce myofibroblast differentiation. This information can also be used by other investigators to guide future research on DNA methylation and $PM_{2.5}$ exposure.

Our Luminex experiments showed that IL-12 is upregulated in fibroblasts following exposure to low concentrations of $PM_{2.5}$. Future experiments could investigate the role of IL-12

in $PM_{2.5}$-induced myofibroblast differentiation further. Additionally, any further attempts to assess cytokine secretion should consider the high degree of experiment-to-experiment variability observed in our attempt here, and adjust accordingly.

6.5 Aim 4 Summary and Future Plans

The objective of Aim 4 was to recapitulate our *in vitro* results *in vivo*. We specifically chose to expose mice to two doses of $PM_{2.5}$ separated three days apart to mirror the conditions that caused myofibroblast differentiation *in vitro*. After six days, we then exposed mice to bleomycin, a common agent that further induces fibrosis. In one flight of the experiment, there was a trend toward worse fibrosis in the bleomycin group that was pre-exposed to $PM_{2.5}$. However, we had some difficulty replicating this result in a follow-up experiment. Differences in the sex of the mice in each experimental flight may have contributed to this variability. In the future, we will account for the effect that sex appears to have on susceptibility to $PM_{2.5}$. Additionally, we will consider varying concentrations of $PM_{2.5}$ and the timing of the exposure relative to bleomycin treatment. These adjustments will be made to attempt to replicate the results of the pilot experiment and not the unsuccessful follow-up. Regardless of the outcome however, a third experiment would help us to definitively determine whether $PM_{2.5}$ exposure *in vivo* results in worse bleomycin induced fibrosis.

6.6 Public Health Implications and Final Thoughts

Throughout this dissertation, we have demonstrated that $PM_{2.5}$ causes fibroblasts to differentiate into myofibroblasts, secrete BMP2, and undergo a myriad of changes in DNA methylation and secretion of other cytokines. All of these changes potentially have the ability to promote fibrosis and lead to diseases such as IPF. $PM_{2.5}$ effects on fibroblasts vary depending on the duration, frequency, and concentration of exposure, demonstrating the public health

importance of even low levels of air pollution. Given the grim prognosis (median survival of 2.5-5 years from diagnosis[178]) and significant societal costs (2-fold increase in healthcare utilization[179]) of pulmonary fibrosis, this study adds to an already large body of literature showing the significant public health costs of $PM_{2.5}$ exposure.

We had previously discussed the associations between $PM_{2.5}$ exposure and negative health outcomes such as mortality and disease incidence[24, 25, 26, 27] but significant economic costs are imparted on society as well. An analysis of Medicare inpatient hospital claims showed that even relatively small, transient increases in $PM_{2.5}$ exposure led to significant increases in inpatient and post-acute care costs while simultaneously causing economic loss through decreases in productivity (illness and premature death)[180]. An economic analysis of data from the European Union found that every 1$\mu g/cm^3$ increase in $PM_{2.5}$ concentration, even at low levels, corresponded to a 0.8% decrease in GDP that same year[181]. What is especially notable about these studies, is that they reveal the economic benefits of improving $PM_{2.5}$ levels even when they are already relatively low.

Furthermore, recent studies have shown that low levels of $PM_{2.5}$, even levels below the standards set by many countries, can have deleterious health consequences. Specifically, reduced pulmonary function[24], and lower birth weight[69] as well as increased risk of hospitalization and death[26, 27], have all been linked to $PM_{2.5}$ exposures at or even below the current $PM_{2.5}$ US National Ambient Air Quality Standards. Even modestly lowering air pollution standards in the United States to account for the effects of low-level exposures would prevent 2800 deaths, and 370 cases of lung cancer while eliminating 12.6 million days of missed school or work. The economic and human health impacts of $PM_{2.5}$ exposure, even exposure to relatively low concentrations, impart significant costs to society. Taken together, the data presented in this

dissertation supports the idea that minimizing $PM_{2.5}$ exposure, even among those who are not highly exposed, would improve overall lung health, promote homeostasis in pulmonary fibroblasts, and reduce overall incidence of pulmonary fibrosis.

Appendix A: Full List of Differentially Methylated Genes from the Low Exposure vs. High Exposure Comparison.

Hypermethylation - Low Exposure vs. High Exposure	
Low	**High**
AAK1	A1CF
ABCB5	AADACL2
ABHD4	ABCA10
ABL2	ABCC5
ACAA2	ABCG1
ACADM	ABCG4
ACCS	ABTB2
ACTN1	ACAP2
ACTR6	ACE2
ACVR2A	ACER2
ADAM12	ACO1
ADAM9	ACOXL
ADAMTS2	ACP6
ADCY9	ACSF3
ADGRG4	ACSL6
ADGRV1	ACSS3
ADH1C	ACTL7A
AFG3L2	ACTR10
AGTR1	ACTR3
AKNA	ADAMTS3
AKR1B15	ADARB2
AKR7A3	ADCK1
ALDH9A1	ADCY8
ALG1L2	ADGRF1
AMD1	ADGRF2
ANKRD11	ADGRF4
ANKRD20A5P	ADNP
ANKRD30B	ADRA1A
ANKRD33B	AFAP1L2
ANKRD45	AGPAT1
ANP32AP1	AHI1

AOC2	AHSA1
AP1B1	AIG1
AP2A2	AIM1L
APC2	AK9
APOBEC4	AKAP10
APOL2	AKIRIN1
APOL4	AKR1B10
AQP4	AKR1B15
AQP4-AS1	AKR1C1
ARHGAP17	AKR1C2
ARHGAP23	AKR1C3
ARHGAP29	AKT3
ARHGAP5	ALDH1L1
ARHGEF12	ALDH1L1-AS2
ARL2BP	ALDH1L2
ARPC3	ALKBH5
ARSK	ALPL
ASAP1	AMER2
ASAP1-IT1	AMN
ASNS	AMY2B
ATAD2	ANGPT1
ATF6B	ANK3
ATP11A	ANKRD1
ATP1B3	ANKS1B
ATP5G2	APBB2
ATP5L	APP
ATP6V0D2	AREG
BACH2	ARF1
BCL7C	ARHGAP22
BRI3BP	ARHGAP25
BTBD16	ARHGEF40
BTN2A2	ARID5B
BTN2A3P	ARL13A
C19orf25	ARL17A
C2CD2L	ARL8B
C6orf203	ARMS2
CABYR	ASB11
CACNB1	ASB3
CAMKMT	ASB5
CAPN14	ASH1L
CBR3-AS1	ATG10
CCDC121	ATG7
CDC20	ATP5J2-PTCD1

CDC27	ATP6V1A
CDH13	ATP6V1B2
CELF4	ATP8A2
CENPJ	ATXN1
CEP112	BCL11B
CEP85L	BCL3
CEPT1	BET1
CFAP44	BET1L
CHCHD3	BICC1
CHMP3	BICDL2
CHST11	BIN2
CIRBP	BIRC6
CLEC14A	BRAF
CLEC18C	BRCA1
CLINT1	BRIP1
COMMD1	BRSK2
COMMD5	BTAF1
COQ4	BTBD1
CRCP	BTBD2
CROCC	BTD
CRSP8P	BTF3L4
CRYGN	BTNL3
CSGALNACT1	C11orf80
CSMD2	C14orf37
CST1	C1QTNF9
CST3	C1orf141
CTGF	C1orf27
CTSO	C2
CUL7	C2CD4A
CUX1	C2CD4D
CXCL17	C3orf30
CXorf36	C5orf34
Cfap43	C6orf15
DAD1	C7ORF26
DAW1	CABP1
DDX60	CACHD1
DEFB108B	CACNA1A
DEGS2	CACNG3
DENND6A	CAMK4
DLEU2L	CAPN7
DLG1	CAPZB
DNAL1	CARD18
DPAGT1	CARD8

DPY19L1	CARD8-AS1
DST	CARF
DUSP12	CASC8
DUSP5	CASKIN1
EFCAB7	CAST
EFL1	CATSPERB
EGR2	CBX5
EHBP1L1	CCDC102A
EHD3	CCDC121
EHMT2	CCDC130
EIF2B3	CCDC144A
EMBP1	CCDC178
ENDOD1	CCDC81
ENDOU	CCNDBP1
ENO1	CCT6A
EP400	CCT8
ERBIN	CCZ1B
ERICH6B	CD160
ERP44	CD2AP
ESYT2	CD300LF
EYS	CDC123
FAF1	CDH13
FAM120A	CDH18
FAM126A	CDH4
FAM156A	CDH5
FAM171A1	CDHR2
FAM174A	CDK14
FAM19A2	CDKL3
FAM45BP	CDKL4
FAM47B	CDSN
FAM49B	CECR1
FAM91A1	CEP104
FAM96A	CEP128
FAM9A	CEP19
FANCD2	CEP41
FAR2	CEP55
FBXO25	CERS6
FBXO32	CES1
FDFT1	CFB
FHOD3	CFH
FKBP5	CHAF1B
FOXJ2	CHMP3
FOXN3	CHN1

FOXP1	CHUK
FSCN1	CISD2
FTX	CKAP2L
FYB	CLEC1B
GABARAPL3	CLLU1
GABPB1	CLLU1OS
GATAD2A	CLRN2
GATS	CMAHP
GC	CNIH3
GGA2	COBLL1
GLRX2	COL23A1
GLT8D2	COL6A1
GNL2	COLGALT1
GOLGA1	COMMD4
GPATCH1	COMMD7
GPHN	COPG2
GPKOW	CORO7
GPN1	CORO7-PAM16
GPR149	CPA5
GRAMD1C	CPNE5
GRIK2	CPSF4
GS1-124K5.11	CPT1A
GTF2A2	CRTC3
GTF2H1	CRYGS
GTF3C6	CRYM
GUCY1A2	CSDE1
GULP1	CSK
GYG1	CSMD3
HADHB	CTBP2
HCFC2	CTNNA1
HDAC3	CTNNA3
HECTD2	CUBN
HECTD2-AS1	CUX1
HERC2	CYP19A1
HEXB	CYP2A7
HGSNAT	CYP4A11
HLA-G	DAB1
HLA-H	DCUN1D3
HLA-J	DEAF1
HMGN1	DENND4C
HOXB6	DET1
HOXB7	DGKI
HPS1	DHDDS

HSPA2	DHX15
HSPA5	DHX29
HTR3E	DKFZP434A062
Hist1h2ag	DLC1
IFI35	DLG2
IFNL3	DLGAP2
IFNL4	DLK1
IL15	DLX6
IL34	DLX6-AS1
INO80	DNAAF2
IPO11	DNAJB6
IPO11-LRRC70	DNALI1
ITGB4	DNMT3A
ITPR2	DOCK1
ITPR3	DOCK9
JUP	DPF3
KANK1	DPH5
KCNG1	DPP4
KCNJ12	DPP6
KCNQ1DN	DPY19L1
KCNQ4	DTX2
KCTD2	DTX4
KIAA1324L	DUSP8
KLC4	DUT
KLHDC4	DYNLT1
KRT42P	EARS2
KRTAP1-4	ECT2L
Krtap1-5	EDF1
LCOR	EEA1
LILRA2	EFCAB14
LINC00310	EFCAB14-AS1
LINC00674	EFNB1
LINC01010	EIF4A2
LINC01091	ELAC2
LINC01128	ELL3
LINC01158	ELOVL5
LINC01569	EML4
LIPE-AS1	ENOX2
LMO7	ENPEP
LNPK	ENTPD4
LOC100131347	EPHA1-AS1
LOC100131626	EPS8
LOC101559451	ERI2

LOC283683	ERLEC1
LOC284344	ERP44
LOC646903	ETAA1
LOC653513	ETNK2
LOC728554	EXOC4
LOC729506	FAHD1
LOC730159	FAM111B
LRBA	FAM117B
LRP5L	FAM133CP
LTBR	FAM151B
LYG1	FAM153A
MAATS1	FAM161B
MACF1	FAM181A
MAFF	FAM181A-AS1
MAML3	FAM200B
MAN1B1	FAM221A
MAN1B1-AS1	FAM221B
MANBA	FAM227B
MAP3K7CL	FAM41C
MAP4	FBN2
MCCC1	FDPSP2
MCM10	FER
MDM2	FER1L6
MEIOC	FGD3
MEIS2	FHL2
MESDC2	FHOD1
METAP1	FIBIN
METTL25	FMN1
METTL5	FN1
MFAP5	FNBP1
MFSD11	FNTA
MIR1185-1	FOXP1
MIR1185-2	FOXR1
MIR300	FRMD4A
MIR3134	FSIP2
MIR376A1	FZD1
MIR376A2	G3BP2
MIR376B	GAB1
MIR4435-2HG	GABBR1
MIR4458	GABPB2
MIR4458HG	GABRA4
MIR4685	GALNT13
MIR548AC	GALR3

MIR548AN	GANC
MIR5690	GAS7
MIR663AHG	GATA4
MKL2	GBX1
MORN3	GCM1
MRPL16	GFI1
MRPL2	GFM2
MRPL30	GGACT
MRPS36	GGN
MTCH1	GJB3
MTIF2	GKN1
MTPAP	GLB1
MYLK	GLS
MYLK-AS1	GLUD1
MYO5A	GLUL
MYT1L	GMDS
Mir654	GORASP2
NACA	GPN1
NAP1L4	GPR132
NAT14	GPSM1
NBPF10	GPT2
NBPF20	GRIK4
NBPF7	GRSF1
NBPF8	GYG1
NBPF9	HACD3
NCF2	HACL1
NCK1	HAGH
NCKAP5	HAND2
NCOA6	HAND2-AS1
NDUFB6	HBE1
NEK6	HBG2
NEMF	HCAR1
NFIX	HCFC1
NIF3L1	HCFC1R1
NLGN2	HCG4
NMNAT3	HCN2
NOTCH2NL	HDAC4
NOX4	HDAC9
NREP	HECW2
NRXN2	HELQ
NUAK1	HEPHL1
NUBP1	HIF1A
NUDCD3	HLA-DMA

NUDT5	HLA-G
NUTM2G	HLA-H
OPA1	HLA-J
OPTN	HMBOX1
OR1D2	HMGCS2
OR4C6	HNRNPA1
OR4K5	HNRNPA3
OR7E14P	HPR
OSBPL9	HPSE
PAFAH1B1	HSPA12A
PARD3	HSPA2
PCBP2	HSPB6
PCSK4	HSPG2
PDE4DIP	HTRA1
PDXDC2P	HYPK
PFDN1	ICA1L
PFN1P2	ID3
PFN2	IFNG
PHACTR1	IGDCC3
PIGH	IGFL2
PIGK	IGFLR1
PIGU	IGSF9B
PKM	IK
PKNOX2	IL12B
PLEC	IL12RB2
PLEKHG1	IL15
PLOD1	INO80D
PLXDC1	INPP4A
POLR2D	INPP5A
POU3F4	INVS
PPP1R11	IPO4
PRCC	IPO5
PRIM2	IQCJ-SCHIP1
PRKCE	ISG20
PRPH	ISLR2
PRSS54	ITGA4
PSG1	ITGB2
PSG6	ITPKB
PSG8	JADE1
PSMB2	JAKMIP2
PTCHD3P1	JAZF1
PTEN	KANSL2
PTP4A1	KBTBD12

PTPRN2	KCNK9
PVRIG	KCNQ1
PYROXD1	KDM3B
QRICH2	KDM5D
RAB11FIP1	KIAA1328
RAB2A	KIF18B
RAB3GAP2	KIF1B
RABGAP1L	KIF6
RACGAP1	KIFC2
RAD54L2	KLF3
RAPGEF4	KLF3-AS1
RASA2	KLHL15
RASSF8-AS1	KLHL41
RBM17	KLRC2
RBM47	KLRC3
RBMS1	KRTAP5-AS1
RCCD1	KTN1
REEP6	LAMA3
RGL1	LAMB1
RGL2	LARP1
RGMA	LBHD1
RHBDF2	LCE3B
RIC3	LCMT1
RNASEH1	LDLR
RNF103-CHMP3	LDLRAD2
RNF145	LEP
RNF157	LHFPL3
RNF215	LHFPL3-AS1
RNF7	LIN37
ROR1	LIN54
ROR2	LINC00271
RPSA	LINC00319
RTN4	LINC00426
SAFB	LINC00523
SDK1	LINC00837
SELENOK	LINC01184
SELENOT	LINC01619
44446	LINC01622
SH3RF1	LIPK
SH3TC1	LMBRD1
SKAP2	LMLN
SKIV2L2	LNX1
SLC16A10	LNX1-AS1

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SLC30A6	LOC100130075
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SLC7A5	LOC100133315
SLIT1	LOC100506207
SMARCC1	LOC101559451
SMC5	LOC149950
SMC5-AS1	LOC283731
SNORA62	LOC283856
SNORA80A	LOC285626
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SNORD114-19	LONRF1
SNX19	LPIN2
SNX29	LRRC27
SNX9	LRRC37A2
SORD	LRRC49
SPARCL1	LRRC59
SPECC1L	LRRC6
SPIDR	LRRTM3
SPN	LSM6
SPR	LYNX1
SSBP4	LYPD5
ST8SIA6	LYRM1
ST8SIA6-AS1	LYST
STAU1	MAEL
STOX1	MAFG
SUMO4	MAFG-AS1
SUSD1	MAGED2
SV2B	MAGI2
SYNGAP1	MANBA
TAB2	MANEA
TACC1	MAP10
TACC2	MAP2
TBC1D3P1-DHX40P1	MAP3K13
TBC1D7	MAP4K3
TBCCD1	MAP7D2
TBPL1	MAP9
TDG	MAPT
TERT	MAPT-AS1

TET1	MAPT-IT1
TFAP2E	MARK4
THBS2	MAU2
TJP1	MAX
TLK2	MBLAC1
TLR2	MBNL1
TMC5	MBOAT2
TMEM109	MCAM
TMEM17	MCAT
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TMEM256	MDM2
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TMEM45B	MEST
TMEM62	MESTIT1
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TNXB	METTL22
TPTE2	METTL7A
TRAK2	MGMT
TRG-AS1	MIER2
TRIM27	MINK1
TRIM4	MIPEPP3
TRIM43	MIR1236
TRPS1	MIR1282
TRUB2	MIR2052HG
TSC2	MIR335
TSEN2	MIR3609
TTC37	MIR3620
TXNDC9	MIR372
TYR	MIR373
TYW1	MIR519A1
TYW1B	MIR522
UBAC2	MIR527
UBB	MIR548AI
UBE2E2	MIR548F1
UBE2V2	MIR548H2
UBE3C	MIR671
UBL3	MKLN1
UBQLN4	MKNK1-AS1
UBTD2	MLLT11
UBXN2B	MME
ULK2	MOB2
UNC80	MORF4L2

URB1	MPHOSPH9
USP10	MRPL13
USP28	MRPS18C
USP34	MRPS25
USP8	MT1A
UVSSA	MT1E
VPS35	MT1JP
VPS54	MTA1
VPS8	MTDH
WDFY3	MTG1
WDFY3-AS2	MTHFD1L
WDR70	MTIF3
WEE2	MTMR2
WEE2-AS1	MTMR3
WHAMM	MTMR9LP
XRCC2	MTR
YAE1D1	MTUS1
YAF2	MYADM
ZBTB45	MYBL1
ZBTB9	MYH2
ZFHX4	MYOM1
ZFHX4-AS1	MYT1L
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ZNF660	NEIL3
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ZNF717	NEMF
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ZNF774	NHEJ1
ZNF781	NLN
ZNRD1ASP	NOL4L
ZRANB3	NOP58

aire	NOTCH3
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bola2	NPHP4
c12orf45	NPTN
c1orf112	NR1H4
cbr3	NR2F2-AS1
cpeb3	NR5A2
cyp2u1	NRADDP
dhx37	NRAS
ecd	NRXN3
epgn	NSA2
fsd1l	NTNG1
il6st	OBSL1
lgals9	OCLM
mbd5	OMA1
meg3	ONECUT1
mta3	OPA1
pelp1	OR1J2
psg3	OR51B2
rubcn	OR6A2
svil	OR6C75
	OR8I2
	OVCH2
	OVOL1
	P3H2
	PAFAH1B2
	PALM2
	PALM2-AKAP2
	PAPOLG
	PARD3B
	PARP4
	PAX6
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	PCBP1-AS1
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	PPT2-EGFL8

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	PRKCA
	PRKCH
	PRKDC
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	PROSER3
	PRPF3
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	PRR4
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	PRSS48
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	PSPH
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	PTPN14
	PTPRD
	PTPRK
	PTPRN2
	PTPRS
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	RESP18
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	SATB2-AS1
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	SIM2
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	SUMF2
	SURF4

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	Snora81
	Snord2
	Snord70
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	TAS2R3
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	TBCE
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	TCF12
	TCF7L2
	TCN1
	TCP11L1
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	TGFBR3
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	THRAP3
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	UBXN4
	ULK4
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	USP50
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	VMP1
	VPS50
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	ZC3H15

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	c4orf45
	c9orf3
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	ccdc17
	chpf2
	clrn3

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	mob1a
	mta3
	nlgn1
	olig1
	pelp1
	plxdc2
	pnkd
	ring1
	slc22a5
	snca
	sorbs1
	stk3
	ttll5

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