Environmental Particulate (PM2.5) Augments Stiffness-Induced Alveolar Epithelial Cell Mechanoactivation of Transforming Growth Factor Beta

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Abstract

Dysfunctional pulmonary homeostasis and repair, including diseases such as pulmonary fibrosis (PF), chronic obstructive pulmonary disease (COPD), and tumorigenesis have been increasing over the past decade, a fact that heavily implicates environmental influences. Several investigations have suggested that in response to increased transforming growth factor -beta (TGFβ) signaling, the alveolar type II (ATII) epithelial cell undergoes phenotypic changes that may contribute to the complex pathobiology of PF. We have previously demonstrated that increased tissue stiffness associated with PF is a potent extracellular matrix (ECM) signal for epithelial cell activation of TGFβ. The work reported here explores the relationship between tissue stiffness and exposure to environmental stimuli in the activation of TGFβ. We hypothesized that exposure of ATII cells to fine particulate matter (PM2.5) will result in enhanced cell contractility, TGFβ activation, and subsequent changes to ATII cell phenotype. ATII cells were cultured on increasingly stiff substrates with or without addition of PM2.5. Exposure to PM2.5 resulted in increased activation of TGFβ, increased cell contractility, and elongation of ATII cells. Most notably, on 8 kPa substrates, a stiffness greater than normal but less than established fibrotic lung, addition of PM2.5 resulted in increased cortical cell stiffness, enhanced actin staining and cell elongation; a result not seen in the absence of PM2.5. Our work suggests that PM2.5 exposure additionally enhances the existing interaction between ECM stiffness and TGFβ that has been previously reported. Furthermore, we show that this additional enhancement is likely a consequence of intracellular reactive oxygen species (ROS) leading to increased TGFβ signaling events. These results highlight the importance of both the micromechanical and biochemical environment in lung disease initiation and suggest that individuals in early stages of lung remodeling during fibrosis may be more susceptible than healthy individuals when exposed to environmental injury adjuvants.

Introduction

Dysfunctional pulmonary homeostasis and repair, including diseases such as pulmonary fibrosis (PF), chronic obstructive pulmonary disease (COPD), and tumorigenesis, have been steadily increasing over the past decade. Many fibrotic pathologies are characterized by excessive extracellular matrix (ECM) deposition, interstitial scar tissue formation, and an increase in tissue stiffness. Specifically, during the course of pulmonary fibrosis, functional lung tissue of the alveoli is replaced with collagen-rich ECM, which leads to rapid and severe decreases in lung compliance and irreversible loss of lung function [1,2]. In addition, another hallmark of PF and other fibrotic conditions is the influx of contractile myofibroblasts. This influx of myofibroblasts further perpetuates the disease through persistent matrix production and contraction. Myofibroblasts are recruited from a variety of sources including local mesenchymal cells, bone marrow progenitors, and via a process of epithelial to mesenchymal transition (EMT), where epithelial cells transdifferentiate into fibroblast like cells. Once these fibroblasts become activated, they transform into myofibroblasts that are capable of secreting ECM components. During wound healing, myofibroblasts lay down a temporary matrix that epithelial cells migrate over to repair the damaged tissue. Fibrosis is thought to occur when this process becomes dysregulated, resulting in persistent matrix production and the formation of a scar.

Lack of effective treatment options for this disease, and other fibrotic pathologies is largely due to the lack of understanding of the exact mechanisms that initiate and propagate fibrosis, however mounting evidence suggests that apoptotic signaling of the lung “precursor” cell, the type II alveolar epithelial cell (ATII), contributes to the initiation and progression of these diseases [3–
ATII cells are pseudo cuboidal, multifunctional cells that are considered the “protector of the alveolus” due to their central role in defense and repair. ATII cells act as the primary surfactant secreting cells, precursors to ATI cells, and in many cases as non-professional antigen presenting cells [6]. These various functions underscore the importance of these cells in maintaining pulmonary function. During normal repair, ATII cells are thought to proliferate, migrate onto a provisional matrix and differentiate into ATI cells. However, recent evidence has suggested that repeated injury of ATI cells may be an underlying contributor of pulmonary fibrotic disorders [8,9]. Specifically, ATII cells can change their phenotype in response to several stimuli including active transforming growth factor beta (TGFβ), tumor necrosis factor alpha (TNFα), epidermal growth factor (EGF), and reactive oxygen species (ROS), as well as in response to ECM proteins including fibronectin (Fn) [5,10–17]. The role of TGFβ is most well defined of all these factors and has been highly implicated in the onset and progression of fibrosis by inducing ATII cell EMT as well as through activation of resident fibroblasts. TGFβ induced EMT may directly contribute to the disease by increasing the number of mesenchymal, ECM secreting cells. Additionally, ATII TGFβ signaling to neighboring fibroblasts may result in their activation, again increasing the number of ECM secreting myofibroblasts. Although the exact mechanisms of action are still unknown, it is under agreement that TGFβ is a major player, and thus understanding what factors lead to increased TGFβ activation and signaling may provide new insights to the development of treatments for PF.

TGFβ must be activated in order to bind to its receptor(s), which can be performed through several different mechanisms including the presence of ROS, proteolytic cleavage, decreased pH, and physical forces [17–34]. The ability of ROS to activate TGFβ is of particular interest in the lung due to the continued exposure of the lung to insults of injury, including environmental particulate matter (PM). Environmental PM is capable of depositing in the airways, penetrating into the alveoli, increasing respiratory distress, and then exacerbating pre-existing pulmonary conditions. Previous studies have highlighted the potential role of PM exposure in predisposing patients to asthma, bronchitis, COPD, and resulting fibrosis [35–38]. The fibrogenic potential of PM is likely due to several factors including particle size, surface area, and composition. Smaller particles in the 1 to 10 micron range have been shown to reach the distal lung, resulting in a higher potential to cause injury due to their high surface to mass ratio [38–40]. Furthermore, particle composition is likely an important determinant of the effect of the PM on the progression of pulmonary disease. Both organic and inorganic agents, such as transition metals, hydrocarbons, and endotoxins can contribute to the composition of the PM [40–42]. Previous studies have shown that many of these components of PM can induce cellular damage, which can stimulate fibrotic remodeling pathways [43–46].

Furthermore, recent evidence indicates that cells sense and respond to the composition and mechanical properties of their underlying ECM. This is of particular interest when studying diseased states, such as PF, where significant changes to both the biochemical and biophysical properties of the ECM have been observed. Specifically, in the case of PF, the ECM is known to shift biochemically from a predominantly laminin (Ln) and elastin matrix to a provisional matrix that is predominantly composed of Fn and eventually collagen. These differences in underlying matrix composition have been shown to have a dramatic effect on the phenotype of alveolar epithelial cells, where Ln maintains an epithelial phenotype and Fn drives EMT [5,47–51]. Additionally, cells are extremely sensitive to their micromechanical environ-

**Materials and Methods**

**Cell Isolation and maintenance**

RLE-6TN cells, an alveolar type-II epithelial (ATII) cell line, were purchased from ATCC and maintained in DMEM/F12 media with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) and incubated at 37°C at 5% CO2. Media was refreshed every 48 hours and cells were split upon reaching 95% confluency.

**Poly-acrylamide gel production**

Poly-acrylamide (PA) gels of varying bisacrylamide concentrations were created on amino-silanated coverslips as previously described [68]. PA gel solutions were produced by combining acrylamide and bisacrylamide to final concentrations of 8% acrylamide (Biorad, Hercules, CA, USA) and 0.048%, 0.117%, 0.208%, or 0.260% bisacrylamide (Biorad) to obtain gels with final elastic moduli of 2 kPa, 8 kPa, 16 kPa, or 24 kPa, respectively. Fifty (50) µl of each solution was polymerized by the addition of ammonium persulfate (VWR, West Chester, PA, USA) and N,N,N',N'-tetramethylethylenediamine (Biorad, Hercules, CA, USA) at different concentrations, followed by overnight incubation with the Fn, gels were washed three times with PBS. Fn was covalently attached to the surface using the heterobifunctional crosslinker sulfosuccinimidyl-6- (4’-azido-2’ nitrophenyl-amino) hexanoate (sulfo-SANPAH; Pierce Chemical Co., Rockford, IL., USA). Following an overnight incubation with the Fn, gels were washed three times with PBS.

**Fine particulate matter isolation**

Fine particulate matter samples were collected on Teflon filters from the Atlanta area (Dekalb county) as part of a study for the Georgia Tech Civil and Environmental Engineering department. The samples were collected daily using multichannel particle...
RLE-6TN cells were maintained and passaged in DMEM/F12 with a 1:100 dilution of 100 μg/mL active TGFβ, but are incapable of activating TGFβ. After 5 days of RLE-6TN culture on the various substrates, MLECgs were added at a density of 50,000 cells/cm² on top of the RLE-6TN cells in serum free DMEM/F12 media +1% BSA. Cells were co-cultured for 16 hours, lysed and luciferase activity was determined via the One-Glo luciferase assay (Promega). To determine total levels of TGFβ, samples were heated to 85°C for 10 minutes prior to plating of MLEC cells. To ensure PM alone did not activate TGFβ, soluble TGFβ was added along with PM as a control. In order to determine if substrate stiffness alone affected the sensitivity of the MLEC cells to active TGFβ, MLEC were cultured for 16 hours with the addition of 200 pg/mL active TGFβ and analyzed for changes in luminescence. To determine the role of contractility, RLE-6TN cells were cultured with or without the addition of PM in the presence of 10 μM Y-27632 (EMD Biosciences). Additionally, to determine the effect of ROS on TGFβ activation, cells were cultured in the presence of 5 μM NAC. Luminescence was measured with a Synergy H4 Multi-Mode Plate Reader (BioTek, Winooski, VT, USA). Luminescence values were normalized to MLEC cultured in the absence of TGFβ. Levels of active or total TGFβ activation were then calculated through interpolation using a standard curve. Results presented are from 3 independent triplicate experiments.

Immunofluorescence staining and cell shape analysis

Following culture for 5 days, cells were washed with PBS, fixed with 4% formaldehyde, permeabilized with 0.2% Triton-X 100 and then blocked with 10% goat serum. To characterize cell shape, actin was stained with Texas-red phalloidin (Invitrogen) and nuclei were stained with Hoescht stain (Invitrogen). Images were acquired with a Nikon Eclipse (TiE) inverted fluorescence microscope at 20× magnification (PlanFluor 20X, 0.5 NA objective) with a CoolSNAP HQ2 Monochromatic CCD camera. Images were processed in triplicate, and images presented are representative from 5–10 random fields for each independent experiment. Images were quantified by determining the total intensity of phalloidin staining in Image J and then normalizing to the number of nuclei per image. To characterize circularity, area and perimeter of individual cells stained for actin were determined for each condition using Image J (NIH Freeware) image processing software, then circularity was determined using the equation circularity = 4π(area/perimeter²). Three independent images were analyzed for each condition, and at least 10 cells were analyzed per image. Data is pooled from all 3 images analyzed per condition.

Atomic force microscopy (AFM) nanoindentation analysis

RLE-6TN cells with or without the addition of each of the three concentrations of PM2.5 were cultured on 8 kPa gels in a 0.17 nm thick glass-bottom petri dish (World Precision Instruments, Inc.). Using the Asylum MFP-3D-BIO AFM, single force points were measured from at least 5 peri-nuclear regions that were greater than 300 nm in height by force spectroscopy using contact mode at a scan rate of 1.2 Hz. The silicon nitride AFM tip (Vecco) was customized with a 4.74 μm diameter polystyrene bead to allow for imaging at appropriate resolution scales and for elastic modulus determination using the Hertz-contact model. Single cantilever’s unique spring constants were determined using the thermal resonance frequency method, with values typically ranging 0.1–0.3 N/m. To determine the Young’s modulus of each contact point, the Hertz-contact model for determining the elastic forces between two spheres was used (Eq. 1). Using known values from the AFM measurement (xs is scanner displacement; xc is the cantilever displacement; c.p. is the contact point) and
Exposure to fine particulate matter (PM2.5) [particulate matter with an aerodynamic diameter less than 2.5 μm] has been associated with a number of pulmonary diseases including asthma, bronchitis, COPD and fibrosis. Previous studies have shown that small-inhaled particles, in the micron range, result in more toxic and fibrotic effects on alveolar cells due to their higher surface to mass ratio, which prevents the particles from being cleared from the lungs. In addition, different components of the particles are able to activate cytokines and growth factors, including TGF-β, the known regulator of ATII cell phenotype. To date, no studies have explored the effect of environmental PM in conjunction with substrate stiffness on ATII cell phenotype. We have previously shown that substrate stiffness induces changes to alveolar epithelial cell phenotype and may be a regulator of processes that drive PF 

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\frac{E}{1 - v^2} = \frac{3}{4} \frac{k_{xc}}{R(x_c - x - c_p)^3}
\]

Particulate Matter Isolation and Analysis

PM used for this study was collected from 1-March-2004 to 30-June-2004 using a filter based particle composition monitor (PCM) as part of the longer term Assessment of the Spatial Composition in Atlanta (ASACA) program [71]. The PCM is a 3-channel system that collects 24 hour integrated samples for analysis of ionic, carbonaceous, and metallic species in the PM2.5 size range. The monitor is controlled by a data acquisition system (DAS) that activates sampling, sequences the filters and controls sample flow to a flow rate of 16.7 L/min through each channel. Filters were installed approximately one day prior to sampling and removed one day following exposure to ambient air. Ambient air was drawn through each channel of the monitor and then flowed through denuders for selective removal of gases, a WINS impactor for providing a 2.5 μm size cut, and finally the sampling media. The PCM was mounted approximately 2.5 m above ground [71]. In channel 1, the sampled air was passed through onto a single Teflon filter. PM2.5 captured on the Teflon filter is archived for exploratory analyses, such as this one. Ionic species including sulfate, nitrate, and ammonium were collected on a nylon filter using channel 2. Finally, channel 3 was used for quantification of elemental and organic carbon. Regular maintenance of the PCM was performed over the collection period.

Particulate matter sample analysis

Concentrations of water-soluble sulfate, nitrate and ammonium, as well as elemental and organic carbon were determined from filter samples collected using the ASACA PCM. In addition, data on these species, as well as from elemental analyses, were available from the nearby (approximately 30 m) monitoring site run by the Georgia Environmental Protection Division (GaEPD), which is also reported as part of the US EPA Air Quality System (AQS). While the ASACA data (and the filters used) are available every day during the period, the GaEPD sampling is done every third day. Table 1 summarizes the average composition of these components from our filters as well as the data reported in AQS. Full data is shown in Supplemental Material. The ionic and carbonaceous composition of the PM obtained from the nylon and Teflon filters shows that the bulk of the PM was sulfate and organic carbon (Table 1), with a significant fraction being elemental carbon. A more complete presentation of the PM composition, including elemental concentrations observed at the GaEPD site and a comparison of the average ionic and carbonaceous PM concentrations for daily (e.g., corresponding to the samples used in the cellular exposure study) versus 1-in-3 day sampling (as used for the source apportionment) is contained in Supplemental Material (Table S1).

Source apportionment modeling of PM2.5

Measurements were used to conduct Chemical Mass Balance (CMB) source apportionment for the days that coincided with the samples used in this analysis as previously described [72] and where GaEPD data was available [i.e., every third day]. The reason for conducting source apportionment when the GaEPD data is available is the need to have elemental species concentrations. The good agreement between the ASACA and GaEPD sampling, and their close proximity, suggest they are sampling from very similar air masses, and that the source apportionment conducted using the concentrations observed at the GaEPD site are applicable for the filters collected as part of the ASACA monitoring. On average, the most prominent sources for the resulting particulate matter were ammonium sulfate and bisulfate, largely due to coal burning in the region, which together comprised 38%, biomass burning at 20%, gasoline-fueled vehicles at 8%, and diesel vehicles at 9%. These results are summarized in Table 2, with full data in Supplemental Material. This source distribution is expected as coal is a major fuel-stock for electricity generation, the sampling site is near two freeways and the March-April months are when prescribed fires are most common in the Southeast.

Results from the particle analysis show that the composition was primarily sulfate, related ammonium, and organic carbon, with
Table 1. Average Comparison of AQS and ASACA Particle Composition for Species of Interest.

|                | SD AQS Data (ug/m3) | ASACA Data (ug/m3) |
|----------------|---------------------|--------------------|
|                | NH4 | NO3 | SO4 | OC  | EC  | NH4 | NO3 | SO4 | OC  | EC  |
| Average        | 1.22| 0.73| 3.86| 4.70| 0.95| 1.40| 0.65| 4.12| 4.92| 0.80|

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Table 2. Average Source Apportionment for South Dekalb PM2.5 Collection.

|        | GV   | DV   | DUST | BURN | COAL | AMSULF | AMBSULF | AMNITR | SOC |
|--------|------|------|------|------|------|--------|---------|--------|-----|
| AVERAGE| 10.01%| 6.86%| 4.70%| 11.06%| 0.83%| 18.69%| 26.64%| 9.06%  | 12.19%|

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smaller amount of elemental carbon, and that the primary sources were those leading to sulfate, biomass burning and mobile sources.

**RLE-6TN cells are viable up to a concentration of 10 µg/cm² of PM2.5**

RLE-6TN cells were plated in 6 well plates at a density of 100,000 cells/cm² and allowed to attach for 24 hours, then treated with increasing concentrations of isolated PM2.5 for 24 hours. PM2.5 was isolated from the Teflon filters and diluted in cell culture media to concentrations of 0.01 µg/cm², 0.1 µg/cm², 1 µg/cm², 5 µg/cm², 10 µg/cm², 25 µg/cm², and 50 µg/cm². These concentrations were picked based on previous literature suggesting these concentrations are generally within physiologically relevant ranges and that anything over 100 µg/cm² has been shown to be toxic to cells. After culture for 24 hours, the cells from each PM2.5 concentration group were trypsinized and resuspended in a 1:1 suspension with trypan blue. The suspension was analyzed by counting dead cells, which were stained blue, and normalizing to the total number of cells in each group.

As shown in Figure 1, over 99% of cells were viable up to a concentration of 10 µg/cm². When the concentration was increased to 25 µg/cm² and 50 µg/cm², cell viability began to decline rapidly to only 63% and 56% of viable cells, respectively. From these viability results, a low, medium, and high concentration of PM2.5 exposure were chosen to explore in further studies. PM2.5 was isolated from the Teflon filters and added to cell culture media at 0.1 µg/cm², 1 µg/cm², and 10 µg/cm² for each of the following described experiments. These concentrations closely mimic environmental exposures in rural (farm), urban city (Atlanta), and metropolis (Beijing) as reported by the World Health Organization.

**Addition of PM2.5 results in elongated cells and decreased circularity**

In order to explore how exposure of RLE-6TN cells to PM2.5 in a range of different elastic moduli environments affects cell morphology, cells were cultured on PA gels of varying substrate stiffness (2–24 kPa) with or without the addition of PM2.5 at 0.1 µg/cm², 1 µg/cm², or 10 µg/cm² for five days. This range of substrate stiffness was chosen based on previous literature that shows that ATII cells experience significantly greater stiffness in fibrotic versus normal lung, with an average Young's modulus of normal lung at approximately 2 kPa versus fibrotic lung at approximately 18 kPa [27,73]. Additionally, during remodeling observed in PF, the underlying matrix shifts from a predominantly Ln and elastin matrix to one that is composed predominantly of Fn. Therefore to most closely mimic this environment, each of our PA gels was coated with Fn. Furthermore, Ln-coated glass, known to maintain epithelial phenotype, and Fn-coated glass, known to induce EMT, were used as controls as previously described [27,29,51]. The cells were then fixed and stained with Texas Red-X conjugated phalloidin to visualize any changes in the actin cytoskeletal organization. Cells cultured on soft substrates and on Ln-coated glass without the addition of PM2.5 displayed the typical rounded epithelial morphology and diffuse cortical staining for actin (Figure 2A, Q).

As substrate stiffness was increased, cells displayed a subsequent elongated morphology and thick, aligned actin filaments (Figure 2I, M). In addition, when PM2.5 was added to the cultures, most significantly at the 10 µg/cm² concentration, cells exhibited enhanced staining of actin filaments. Notably, cells cultured on 8 kPa gels showed a considerable increase in actin fibers and cell elongation with the addition of PM2.5 when compared with matched substrate-stiffness controls (Figure 2, E-H). This substrate stiffness is of particular interest because it most closely matches that of slightly fibrotic lung tissue, indicating that PM2.5 may have more marked effects on a lung that is undergoing fibrotic remodeling. In addition, this increase appeared to be concentration dependent, with slightly more cell elongation and stress fiber formation seen at the 10 µg/cm² concentration compared to 1 µg/cm² and 0.1 µg/cm² concentrations. Total fluorescent intensity of each image was calculated, normalized to the total number of nuclei, and reported in the bottom right corner of each image (Figure 2A-R). Additionally, cell circularity was calculated to quantify differences in the observed cell shape. Values closer to 1 indicate a more rounded, epithelial like cell morphology.

**Figure 1. RLE-6TN cells are viable up to a concentration of 10µg/cm².** (A) Schematic illustrating process of PM2.5 collection for cell culture experiments. (B) RLE-6TN cells were cultured with increasing concentrations of isolated PM2.5 for 24 hours and analyzed using the trypan blue assay. Approximately 100% of cells were viable through a concentration of 10µg/cm². Treatment with concentrations above 10µg/cm² resulted in significant cell death. Significance shown for * (p<0.01) doi:10.1371/journal.pone.0106821.g001
Statistical significance is shown for each experimental group in comparison to its same substrate stiffness group with no PM2.5 added. These results suggest that the addition of PM2.5, most notably at the 10 μg/cm² concentration result in a more elongated cell indicated by the decreased values of cell circularity.

Increased cell elongation and stress fibers coincide with increased cell stiffness

Based on the observation that PM2.5 resulted in decreased cell circularity and increased actin fiber staining; most notably at the 8 kPa substrate stiffness, it was investigated if these phenotypic observations coincided with an increase in cell stiffness. Previous studies have linked increased epithelial cell stiffness in increased substrate stiffness environments that are commonly seen during PF [27]. Furthermore, several studies have linked increased cell stiffness with increased cell contractility and TGF β activation [27].

Therefore, to explore if the addition of PM2.5 increased cell stiffness, RLE-6TN cells were cultured on Fn-cross-linked 8 kPa gels with the addition of 10 μg/cm², 1 μg/cm², or 0.1 μg/cm² concentrations of PM2.5 and changes in the actin cytoskeleton were analyzed by phalloidin staining of actin filaments and quantified as mean fluorescent staining of actin per cell (A-R). Single cell cortical stiffness of RLE-6TN cells cultured on 8 kPa gels were measured by AFM and significance shown for each group compared to the No PM2.5 control (S). Cell circularity was calculated from acquired images. Values closer to 1 indicate a more rounded, epithelial like cell (T). Experiments were performed in triplicate, representative images are presented and significance shown for * (p<0.05), ** (p<0.01) and *** (p<0.001).

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(Figure 2T). Statistical significance is shown for each experimental group in comparison to its same substrate stiffness group with no PM2.5 added. These results suggest that the addition of PM2.5, most notably at the 10 μg/cm² concentration result in a more elongated cell indicated by the decreased values of cell circularity.

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Figure 2. Exposure to PM2.5 results in elongated cell phenotype and increased cell cortical stiffness. RLE-6TN cells were cultured on Fn-PA gels or Fn- or Ln-coated glass for 5 days with the addition of 10μg/cm², 1μg/cm², or 0.1μg/cm² concentrations of PM2.5, and changes in the actin cytoskeleton were analyzed by phalloidin staining of actin filaments and quantified as mean fluorescent staining of actin per cell (A-R). Single cell cortical stiffness of RLE-6TN cells cultured on 8 kPa gels were measured by AFM and significance shown for each group compared to the No PM2.5 control (S). Cell circularity was calculated from acquired images. Values closer to 1 indicate a more rounded, epithelial like cell (T). Experiments were performed in triplicate, representative images are presented and significance shown for * (p<0.05), ** (p<0.01) and *** (p<0.001).
Alveolar epithelial cells display increased TGFβ activation with the addition of PM2.5

Previous studies have shown that elevated levels of cell stiffness coincide with increased cell contractile forces, ultimately enabling the activation of latent TGFβ [27,51]. Since we saw increases in cell-stiffness with the addition of PM2.5, we investigated if the addition of PM2.5 also resulted in differences in TGFβ activation, which could lead to further changes in ATII cell phenotype. To determine if addition of PM2.5 to alveolar cells on differing substrate stiffnesses induced TGFβ activation, the mink lung epithelial reporter cell (MLEC) bioluminescence co-culture assay was performed for each PM2.5 concentration. In order to confirm that substrate stiffness alone did not alter the MLEC sensitivity to active TGFβ, MLECs were cultured for 16 hours on each substrate stiffness with the addition of 200 pg/ml active TGFβ and analyzed for changes in luminescence. Despite the changes in underlying substrate, the MLECs remained equally sensitive to the fixed amount of active TGFβ (Figure S1). Similar to the cell morphology experiments, RLE-6TN cells were cultured on PA gels ranging from 2–24 kPa with or without the addition of each concentration of PM2.5. In controls without the addition of PM2.5, alveolar epithelial cells were found to increasingly activate TGFβ in response to increases in substrate stiffness. As expected, the control groups cultured on Fn-coated glass exhibited high levels of TGFβ activation, while those cultured on Ln-coated glass showed significantly less levels. When PM2.5 was added to the cultures for 5 days, the cells were found to activate significantly greater amounts of TGFβ in a dose dependent manner (Figure 3).

Of particular interest was the comparison of TGFβ activation across the PM2.5 concentrations at 8 kPa, a physiologically relevant stiffness during the progression of human lung fibrosis. At this substrate stiffness with the addition of PM2.5, we observed an increase in TGFβ activation similar to what was observed on high substrate stiffness and Fn-coated glass without the PM2.5. PM2.5 addition to a solution of inactive TGFβ was found insufficient to activate TGFβ (Figure 3, MLEC control), strongly suggesting that increases in TGFβ activation observed

![Graph showing TGFβ Activation](image)

**Figure 3.** Stiffness-mediated activation of TGFβ is increased by addition of PM2.5. RLE-6TN cells were cultured for 5 days on substrates of increasing stiffness with or without the addition of each concentration of PM2.5 and levels of TGFβ activation were determined using the MLEC bioluminescence co-culture assay. Statistical significance is shown for each concentration of PM2.5 within its same substrate stiffness (*p<0.0001). doi:10.1371/journal.pone.0106821.g003

are mediated by the cells. Statistical significance is for each experimental group compared within its same substrate stiffness.

Exposure to PM2.5 results in increased TGFβ activation without cell contractility

The results thus far have indicated that exposure of RLE-6TN cells to PM2.5, most significantly at concentrations of 10 µg/cm², result in significant changes to ATII cell phenotype. Briefly, ATII cells exhibit enhanced actin filament staining, cell stiffness, and TGFβ activation. These changes are likely dependent on cell contractility, allowing for a mechanically driven activation of TGFβ that subsequently drives the observed phenotypic changes [25–27,29,33,34,65]. Therefore, the MLEC assay that measures TGFβ activation was performed again with the same protocol but with the addition of the ROCK contractility inhibitor Y-27632 during the 5-day culture.

In the presence of the contractility inhibitor, Y-27632, cells that were not treated with PM2.5 activated low levels of TGFβ regardless of the substrate stiffness they were cultured on. However, there were significant increases in TGFβ activation with the addition of 10 µg/cm² PM2.5 on all substrate stiffnesses (Figure 4). These results suggest that inhibition of cell contractility is sufficient to inhibit substrate mediated TGFβ activation, but not sufficient to inhibit TGFβ activation mediated by the exposure to PM2.5. A control where PM2.5 was added to a solution of inactive TGFβ was found insufficient to activate TGFβ (Figure 4, MLEC control), again suggesting that these increases in TGFβ activation observed due to PM2.5 are still mediated by the cells, just not by cell contractility. This data suggests a second, non-mechanical mechanism of TGFβ activation that occurs when the cells are exposed to PM2.5.

Exposure of RLE-6TN cells to PM2.5 results in increased levels of ROS

It was hypothesized that the observed additional activation of TGFβ when cells were exposed to PM2.5 was due to intracellular production of ROS, ultimately leading to increased TGFβ activation [24,74]. In order to explore this hypothesis, RLE-6TN cells were cultured on PA gels of increasing substrate-stiffness with or without exposure to 10 µg/cm² of PM2.5 and analyzed by the DCFH2-DA assay, which measures total levels of intracellular
When RLE-6TN cells were cultured on increasing substrate stiffnesses in the absence of PM2.5 there was a significant increase in ROS solely due to the underlying substrate, supporting previous studies [72] (Figure 5A). Interestingly, the levels of ROS at 8 kPa, the stiffness most closely matching a remodeling lung, were not significantly higher than those seen on a soft substrate. However, when the cells were cultured with the addition of PM2.5, there was a significant increase in ROS observed on all substrates, including Fn- and Ln-coated glass (Figure 5B). This suggests that increased tissue stiffness commonly seen in PF patients likely results in increased intracellular ROS levels and that exposure of the distal lung to PM2.5 significantly increases these levels of ROS. Furthermore, the data show similar levels of ROS across all substrate stiffnesses when treated with PM (Figure 5B). Given that the measured fluorescence values were well within the range of detection for the assay, this data suggest that the cells may only be capable of producing a finite level of ROS, and that this maximum was approached.

Additionally, several studies to date have suggested antioxidants including N-acetyl-cysteine (NAC), may be effective in decreasing levels of ROS within lung tissue [73–77]. Therefore, it was explored if treatment with NAC was able to decrease the ROS production observed due to the exposure of cells to PM2.5. Similar to the previous experiment, RLE-6TN cells were cultured on increasing substrate stiffnesses with or without the addition of PM2.5. Additionally, groups with PM2.5 added were treated with 5 μM NAC. For each of the experimental groups, treatment with NAC abolished the levels of intracellular ROS observed with exposure to PM2.5 (Figure S2). Notably, on higher substrate stiffnesses, i.e. 16 and 24 kPa, these antioxidants were able to reduce the ROS levels below the levels seen without the addition of PM2.5. This suggests that substrate stiffness alone is capable of inducing increases in intracellular ROS, and that this effect can be mitigated by treatment with an antioxidant.

Stiffness-mediated ROS production is TGFβ dependent

The previous data suggest that the levels of ROS increase as a function of matrix rigidity in the absence of treatment with PM2.5. Given previous literature that has shown an intimate link between ROS and TGFβ [24,74,78], we explored if this increase in ROS was TGFβ dependent. RLE-6TN cells were cultured with or without 10 μg/ml PM2.5 as well as with 10 μg/ml of a TGFβ neutralizing antibody for five days and analyzed for ROS production by the DCFH2-DA assay. When cells were cultured in the absence of PM and exposed to the TGFβ neutralizing antibody, the level of ROS production was reduced to baseline (i.e. Fn-2 kPa and Ln-glass) (Figure 6), indicating that the increase in ROS due to substrate stiffness is a result of increased levels of active TGFβ. Additionally, when cells were treated with PM2.5 in conjunction with the neutralizing antibody there was a significant decrease in intracellular ROS, but not to the level seen without PM. Notably, this effect is most prominent at the lower substrate stiffness (2 kPa and 8 kPa), further suggesting that the cells may reach a maximal possible level of ROS production.

Combinatorial treatments diminish increased levels of TGFβ activation

Previous data and the experiments thus far have strongly suggested that increased substrate stiffness is able to induce cell contractility leading to mechanical activation of TGFβ, resulting in downstream changes to ATII cell phenotype. Furthermore, when alveolar epithelial cells are exposed to PM2.5 there were further increases observed in TGFβ activation beyond that seen on increased substrate stiffness alone. Finally, exposure to PM2.5 resulted in increased intracellular ROS levels, which could be mitigated, but not abolished by a neutralizing TGFβ antibody. Therefore, we sought to determine if treatment with NAC could decrease the levels of TGFβ activation to a level seen on Ln-coated glass. Importantly, active TGFβ is a disulfide-linked homodimer, and NAC is capable of reducing disulfide bonds. Therefore, we first evaluated any differences in MLEC sensitivity to active TGFβ.
when also exposed to NAC. MLEC were cultured for 16 hours with a 200 pg/ml TGFβ and 5 μM NAC combination and demonstrate a small, nonsignificant, but consistent decrease in the level of luciferase. When treated with 200 pg/ml active TGFβ and NAC, the luciferase reading corresponded to an approximate level of luciferase. When treated with 200 μg/ml TGFβ, increased antioxidants and blocked cell contractility was able to demonstrate a small, nonsignificant, but consistent decrease in the activation. It was observed that a combinatorial treatment that conjunction with NAC could completely reduce levels of TGFβ despite the substrate stiffness of Fn-coated PA surfaces (Figure 7C). These results taken together strongly suggest that increased tissue stiffness results in increased cell contractility which mechanically activates TGFβ; while exposure to PM2.5 further increases this TGFβ activation by increasing intracellular ROS. These events appear to have an additive effect on the activation of TGFβ, despite the underlying substrate stiffness and result in the significant changes observed in ATII cell phenotype.

Discussion

TGFβ has been implicated as a major regulator during the progression of invasive pathologies such as PF. Therefore, it is imperative to understand how changes in the microenvironment during different pathological conditions affect TGFβ in order to give insight for future therapeutic options. Several studies have previously shown that the micromechanical environment that alveolar epithelial cells and resident fibroblasts interact with is a strong driving force for activating TGFβ. Here we show that in addition to increased tissue stiffness, exposure to environmental stimuli, such as PM2.5, is involved in further increasing TGFβ and driving changes to alveolar epithelial cell phenotype. Here we observe that PM2.5 is involved in the increase in epithelial cell cortical stiffness, an indirect measure of cell contraction, which enables mechanical activation the cytokine TGFβ. Our data show that increases in epithelial cell cortical stiffness coincide with increased levels of TGFβ activation, and an increase in elongated cells that exhibit enhanced actin staining. Experiments were performed where cell contractility was blocked by the ROCK inhibitor Y-27632 and TGFβ activation was measured by the MLEC assay. Interestingly, when cells were treated with 10 μg/cm² PM2.5, inhibition of cell contractility was able to decrease TGFβ activation levels, but not to the level seen without PM2.5 exposure. This indicates that there is a second mechanism, independent of cell contraction, with which TGFβ is activated in response to PM2.5 exposure.

The alveolar epithelium forms a continuous, highly regulated physical barrier, which serves as a protector against inhaled environmental agents, including PM2.5. When epithelial cells become elongated, the tight cell junctions commonly seen in the epithelium are lost, likely resulting in decreased protection against inhaled agents. PM2.5 has been implicated in increasing levels of ROS and previous studies have shown that the release of ROS can activate TGFβ, [38,43,45,79]. Additionally, TGFβ itself is known to be able to induce ROS production as part of its signal transduction pathway. Therefore, ROS were determined to be a likely culprit for the further increase in TGFβ activation that was observed that could not be abrogated by the inhibition of cell contractility. First, total levels of intracellular ROS with or without

Figure 6. Stiffness mediated ROS production is TGFβ dependent. RLE-6TN cells were cultured on PA gels of increasing stiffness with or without the addition of 10 μg/cm² and exposure to a TGFβ blocking antibody for 5 days. Levels of intracellular ROS were measured by the DCFH2-DA oxidation and assay. Three independent triplicate experiments were performed and statistical significance is shown between the PM2.5 groups with or without the TGFβ antibody. *** (p<0.001)
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the addition of 10 μg/cm² PM2.5 were measured on substrate stiffnesses ranging from 2 to 24 kPa. Significant increases in ROS were observed on all substrate stiffnesses when cells were exposed to PM2.5. Interestingly, as substrate stiffness was increased, there was a decreased difference between the non-treated and PM2.5 added groups, showing that substrate stiffness alone has an effect on increasing ROS levels. Furthermore, when cells were exposed to PM2.5, the levels of ROS production were similar despite the underlying matrices, suggesting that the cells may have a finite level of ROS they are capable of producing, and that this level was approached. We explored if the increase in ROS due to matrix rigidity alone was a function of increased amounts of active TGFβ by repeating the ROS assay in the presence of a TGFβ neutralizing antibody. It was shown that neutralizing TGFβ could eliminate the increased ROS due to substrate rigidity, but not due to exposure to PM2.5. Next, it was explored if treatment with the common antioxidant, NAC, could decrease the levels of ROS observed with PM2.5 exposure. When the cells were treated with NAC in conjunction with PM2.5 exposure, ROS levels were significantly decreased. Notably, on stiffer substrates, the antioxidant treatment decreased intracellular ROS to levels lower than seen on the matched substrate stiffness without the addition of PM2.5. This data further confirmed that substrate stiffness alone is capable of increasing levels of ROS.

A previous pulmonary fibrosis drug trial utilized treatment with NAC showed some benefit in delaying the progression of PF, but was not able to restore lung function or reverse any damage currently present [39]. From the data presented here, we conclude that NAC treatment alone was not a fully successful treatment since it has clearly been shown that the mechanical environment is a crucial mediator of TGFβ activation, alveolar cell phenotype changes, and the onset of a fibrotic scar. Therefore, in a final experiment, it was explored if combinatorial treatment with the antioxidant NAC in conjunction with the ROCK contractility inhibitor, could mediate the significantly increased levels of TGFβ activation previously observed with epithelial cells cultured on increased substrate stiffness and exposed to PM2.5. When alveolar epithelial cells were cultured with PM2.5 and treated with NAC as well as Y-27632, levels of TGFβ were decreased to levels seen from cells cultured on Ln, the known epithelial maintaining substrate. This level of TGFβ activation was seen despite the stiffness of the substrate that cells were cultured on. This final piece of data strongly suggests that TGFβ activation is mediated by a combination of mechanical stimuli, i.e. tissue stiffness, and changes in the intracellular levels of ROS.

This work shows that alveolar epithelial cells exhibit increased cell stiffness, TGFβ activation, and changes in their phenotype in response to PM2.5 exposure. These results highlight the importance of studying not only the micromechanical environment in lung disease but also the exposure to environmental injury adjuvants in diseased lung models. Previous findings have indicated that increased tissue stiffness is able to lead to increased cell contraction and TGFβ activation and represents one of the initial stages of pulmonary fibrosis [16,18,25,26,34,70]. Here these findings are expanded and show that in addition to the delicate balance between ECM stiffness and TGFβ, that exposure to environmental stimuli such as PM2.5 enhances TGFβ activation through increasing levels of intracellular ROS. Specifically, it appears that there is an additive effect on TGFβ activation from mechanical stimuli and intracellular ROS production in response to environmental stimuli, resulting in significantly increased changes to alveolar epithelial cell phenotype.

Finally, it is shown that by controlling both the micromechanical and biochemical environment by blocking cell contractility and reducing intracellular levels of ROS, that TGFβ activation can be reduced to levels typically seen in ‘healthy’ epithelial cells. This work suggests that PM2.5 is able to significantly increase the levels of TGFβ activation and may have a role in driving a pre-existing fibrotic phenotype in pulmonary cells. Therefore, approaches to treat pulmonary fibrosis must focus on controlling both the micromechanical stimuli presented to alveolar epithelial cells while also controlling the oxidative effect of increased ROS. Future work should focus on further elucidation of the pathways that both tissue stiffness and PM2.5 engage to initiate changes to alveolar cell phenotype and additionally how these pathways intersect in order to develop novel treatment therapies for pulmonary fibrosis.

**Supporting Information**

**Figure S1** MLEC are equally responsive to active TGFβ on increasing substrate stiffnesses. MLEC were cultured for 16 hours on substrates of increasing stiffness for 16 hours with the addition of 200 pg/ml active TGFβ and analyzed for any...
changes in luciferase response. (A) Three independent triplicate experiments were performed. (TIF)

Figure S2  Addition of the antioxidant NAC restores low levels of ROS. RLE-6TN cells were cultured on increasing substrate stiffnesses with the addition of 200 pg/mL active TGFβ and 5 μM NAC and analyzed for luciferase response. Three independent triplicate experiments were performed. (TIF)

Table S1  South Dekalb Elementary Analysis. Filters collected on given dates were analyzed for specific elemental analysis and are reported in μg/m³. (TIF)

Author Contributions
Conceived and designed the experiments: MD AR TB. Performed the experiments: MD. Analyzed the data: MD BG. Contributed reagents/materials/analysis tools: AR TB. Wrote the paper: MD AR TB.

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