Feedback amplification of fibrosis through matrix stiffening and COX-2 suppression

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Introduction

Fibrosis is a pathobiological process common to many human diseases characterized by the progressive replacement of normal parenchymal tissue with collagen-rich extracellular matrix (Desmoulière et al., 2003). Fibrogenesis occurs through the aberrant accumulation and activation of myofibroblasts, which deposit extracellular matrix components and remodel the tissue (Kuhn and McDonald, 1991; Zhang et al., 1994), culminating in increased tissue mechanical stiffness (Gross and Hunninghake, 2001; Tomasek et al., 2002). In its most severe clinical manifestations, fibrosis can reach a progressive and self-sustaining phase, the cause of which remains largely mysterious.

Much attention has focused on the role that soluble inflammatory and fibrogenic mediators play in the initiation and progression of fibrosis (Wynn, 2008). However, recent observations have highlighted the fact that variations in matrix stiffness potently alter fibroblast morphology, proliferation, TGF-β signaling, and myofibroblast activation (Arora et al., 1999; Wang et al., 2000; Paszek et al., 2005; Goffin et al., 2006; Wipff et al., 2007). These findings raise the intriguing possibility that stiffening of the mechanical environment, long regarded only as an outcome of fibrosis, might play a pivotal role in driving the cellular behaviors that promote, amplify, and perpetuate fibrosis. In the rat liver, macroscale measurements of organ stiffness during onset of carbon tetrachloride–induced fibrosis indicate that stiffening occurs rapidly and may even precede measurable fibrosis, suggesting a possible role for stiffening in the initial stages of fibrosis (Georges et al., 2007). In the lung, fibrotic lesions are heterogeneously distributed, and although it is known that fibrosis alters tissue mechanics (Bachofen and Scherrer, 1967), measurements of stiffness at the local microscale relevant to resident cells are not available. Thus, the role of matrix stiffening in promoting lung fibrogenesis remains untested.

To assess the role that pathophysiological matrix stiffening plays in the promotion of fibrogenesis, we designed experiments to measure the stiffness of normal and fibrotic lung tissue...
and investigated key fibrogenic behaviors of human lung fibroblasts grown on substrates spanning this stiffness range. Our results demonstrate a pivotal role for matrix stiffening in promotion of fibroblast proliferation and matrix synthesis and uncover stiffness-driven suppression of COX-2 (cyclooxygenase-2) expression and PGE$_2$ (prostaglandin E$_2$) synthesis as a key link between matrix stiffening and fibroblast activation. We further show that PGE$_2$ and Rho kinase (ROCK) exert mutually antagonistic effects on fibroblasts, leading to stable and diametrically opposed quiescent and activated states influenced by the stiffness of the underlying matrix. Because matrix stiffening promotes self-reinforcing effects on fibroblast matrix synthesis, our findings show how feedback signaling in the context of mutually antagonistic signaling cascades can switch fibroblasts from a quiescent state to self-sustaining activation supportive of progressive fibrosis.

**Results**

**Stiffness of normal and fibrotic lung tissue**

To establish baseline data on the homogeneity and variance of lung parenchymal tissue stiffness at a scale relevant to resident cells, we prepared thin slices of fresh murine lung tissue and applied atomic force microscopy (AFM) micro indentation to map local elastic properties (Fig. 1). To compare normal and fibrotic tissue stiffness, samples were harvested 14 d after intratracheal administration of either saline or bleomycin. Administration of the latter is well recognized to induce pulmonary fibrosis in a heterogeneous pattern that mimics aspects of human pulmonary fibrosis (Moore and Hogaboam, 2008). Consistent with prior work in mice, bleomycin-treated lungs exhibited focal increases in expression of collagen I (Fig. 1 A) and the myofibroblast marker $\alpha$-smooth muscle actin ($\alpha$-SMA; not depicted; Zhang et al., 1994; Pena et al., 2007; Moore and Hogaboam, 2008). Stiffness mapping was performed in areas devoid of major airways or blood vessels (Fig. 1 A); in bleomycin-treated lungs, mapping was restricted (except where noted) to areas of grossly evident fibrosis.

Elastographs from AFM microindentation mapping demonstrated striking differences in the range and distribution of tissue stiffness in normal and fibrotic lung parenchyma (Fig. 1 B and Fig. S1). The parenchymal tissue from saline-treated lungs was highly compliant (median shear modulus $\sim$ 0.5 kPa), with only rare portions of the mapping area exhibiting stiffness $>3$ kPa. These observations, the first made on intact lung parenchyma at the microscale, are consistent with a long-standing model of lung mechanics in which alveolar walls are assumed to offer negligible contributions to lung elasticity compared with the extracellular matrix–rich connective tissue sheath that connects the alveolar ducts, airways, and pleura (Wilson and Bachofen, 1982). In bleomycin-treated lungs, regions rich in collagen I expression exhibited an overall increase in shear modulus to a median 3 kPa, with highly localized increases in stiffness to $>15$ kPa (Fig. 1 C and Fig. S1 B). These focal changes in tissue stiffness far exceed the two- to threefold increases in stiffness observed previously in tissue strips from bleomycin-treated lungs (Ebihara et al., 2000). Local increases in stiffness were restricted to collagen-enriched regions; when we examined the tissue stiffness of bleomycin-treated lungs in areas devoid of fibrosis, the shear modulus was similar to that observed in saline-treated lungs (Fig. S1 C). Together, the elasticity mapping results emphasize the focal nature of stiffness changes in early fibrotic lesions and establish an unexpectedly large range in tissue stiffness between highly compliant normal lung parenchyma and locally stiffened fibrotic lesions.
Regulation of fibroblast accumulation by matrix stiffness

To elucidate the effects of variations in stiffness on lung fibroblast biology, we designed and fabricated gradient stiffness hydrogel substrates by using photopolymerization of polyacrylamide through gradient masks, an approach adapted from Wong et al. (2003). The resulting hydrogels exhibited a 1D gradient in shear moduli spanning 0.1–50 kPa over a distance of ~6 mm (Fig. S2 A). The stiffness range was selected to encompass and surpass that observed in the murine bleomycin model to account for the increasing stiffness of extensively cross-linked mature fibrotic lesions (Dunn et al., 1985). To permit cell attachment, the inert polyacrylamide surface was derivatized with collagen I using a heterobifunctional cross-linker. The uniformity of the collagen I surface density was confirmed by immunostaining with collagen I primary antibody, followed by secondary antibody conjugated to fluorescently labeled beads that interact only with surface-available antigens (Fig. S2; Pelham and Wang, 1997).

Human lung fibroblasts (CCL-151) attached uniformly across the stiffness gradient, with only modest variability in the degree of cell spreading apparent at 4 h (Fig. 2 A). However, incubation of fibroblasts on these stiffness gradients for 120 h in the presence of 10% FBS gave rise to striking patterns of stiffness-dependent fibroblast morphology and accumulation (Fig. 2 A). Increasing stiffness was accompanied by prominent transitions in fibroblast morphology from attenuated, rounded cells at low stiffness to an appearance marked by multiple dendritic processes at intermediate stiffness (Fig. 2 A). At stiffness levels of 20–50 kPa, fibroblasts aligned in parallel swarms of spindle-shaped cells typical of patterns observed in 3D fibroblastic foci in vivo (Kuhn and McDonald, 1991; Gross and Hunninghake, 2001) and on standard rigid cell culture substrates. The crossover from net cell loss at low stiffness to cell accumulation at high stiffness identified an equilibrium point where cell migration, proliferation, and death were in balance under the given culture conditions (Fig. 2 B). Intriguingly, we observed this intersection to occur reproducibly at shear moduli in the range of 0.5–3 kPa, which is only slightly higher than the median shear modulus measured in normal lung tissue. The pattern of stiffness-dependent accumulation was attenuated when serum was diluted (Fig. 2 C), indicating that both soluble and mechanical factors were necessary elements in the response. However, addition of the fibrogenic cytokine TGF-β1 did not alter the pattern of cell accumulation anywhere along the stiffness gradient (Fig. 2 C). Together, these results suggest that the compliance of the normal lung itself protects against fibroblast proliferation, even in the presence of a growth-promoting or fibrogenic-soluble environment, but that only relatively small changes in stiffness are needed to shift lung fibroblasts into a mode of rapid accumulation.

To elucidate the cellular processes contributing to the stiffness-dependent accumulation of fibroblasts, we compared cell proliferation, apoptosis, and migration across the stiffness gradient. BrdU-positive cells, indicative of ongoing DNA synthesis, were absent from areas of stiffness equivalent to normal median lung stiffness levels even in the presence of 10% FBS but were detected with increasing frequency as substrate stiffness increased (Fig. 2 D and Fig. S3 A). In contrast, serum depletion for 24 h revealed the opposite pattern of staining for active caspases 3/7, end effectors of apoptosis (Fig. 2 D and Fig. S3 B), with decreased staining observed as substrate stiffness increased. Consistent with stiffness-dependent protection from apoptosis, a similar but more muted distribution of active caspase 3/7 was observed across the stiffness gradient in the presence of serum (unpublished data). These results concur with and considerably extend prior findings in 2D (Wang et al., 2000) and 3D (Fringer and Grinnell, 2001) systems by demonstrating that a critical shift in bias from fibroblast apoptosis to proliferation occurs within the pathophysiological stiffness range measured in normal and fibrotic lung parenchyma.

Because cells have previously been shown to preferentially migrate toward stiffer regions when cultured on substrates with sharp stiffness gradients (Lo et al., 2000), we measured lung fibroblast migration at various locations along the stiffness gradient. Although fibroblast migration speed and persistence varied with substrate stiffness (Fig. 2 F), consistent with prior observations using vascular smooth muscle cells (Peyton and Putnam, 2005), no preferential migration relative to the direction of the stiffness gradient was observed (Fig. 2 G). A similar lack of preferential migration has been observed in vascular smooth muscle cells on stiffness gradients of similar slope (Isenberg et al., 2009). Although we cannot rule out a role for stiffness-directed migration in the steeper stiffness gradients found in the intact lung, in the current experiments, the predominant factor in stiffness-dependent accumulation appeared to be the shift in balance between apoptosis and proliferation. Subsequent observations of fibroblast accumulation in wells of discrete stiffness, where migration along a stiffness gradient was not a factor, revealed similar patterns to those observed in the stiffness gradient, which is consistent with a dominant role for proliferation over migration (see Matrix stiffness promotes fibrogenesis through COX-2 suppression).

Regulation of fibroblast matrix synthesis by stiffness

Fibroblasts are the major source for excess deposition of extracellular matrix components, including collagen I, in fibrotic disorders (Hinz et al., 2007). We observed a gradual increase in procollagen I immunofluorescence staining in cells along a continuous stiffness gradient (Fig. 3, A and B; and Fig. S3 C), starting from no detectable procollagen I signal at physiological median stiffness (~0.5 kPa). Analysis of mRNA from fibroblasts grown on discrete stiffness gels demonstrated parallel increases in COL1A1 and COL3A1 transcripts (encoding collagen I[α1] and collagen III[α1] chains) with increasing stiffness and a prominent decrease in expression of MMP1 (encoding collagenase-1; Fig. 3 C). Although stiffness-mediated expression of collagen gene expression has been noted previously (Li et al., 2007), our results extend this observation by demonstrating enhanced procollagen protein expression and parallel decreases in gene expression of a key collagenolytic enzyme with increasing stiffness. Further supporting a matrix synthetic effect of increasing stiffness, we found that net secretion of collagen increased on a per cell basis as matrix stiffness increased on discrete stiffness gels (Fig. 3 D).

Supplementation of culture medium with exogenous TGF-β1 for 3 d shifted procollagen I protein expression to as
Figure 2. **Fibroblasts preferentially accumulate across stiffness gradient.** (A) Human lung fibroblasts stained with phalloidin (red) to visualize F-actin and Hoechst 33342 (blue) to visualize nuclei. Cells attach uniformly across the stiffness gradient 4 h after seeding. After 120 h, fibroblasts accumulate preferentially to stiffest region, and cell morphology transitions from round to spread. Panorama images were generated by imaging the entire gel width along the stiffness gradient and then tiling five to seven adjacent pictures. Arrowheads below the image indicate stitching positions. (B) Cell density is constant across the gradient after 4 h, but by 120 h, cells dramatically accumulate at stiffness >3 kPa and are lost below this stiffness level (note logarithmic scale). Density values are normalized to the global mean at 4 h. (C) Serum but not TGF-β1 is required for stiffness-dependent cell accumulation. Serial dilutions of serum gradually attenuate the stiffness-dependent accumulation behavior (black, 10% FBS; blue, 1% FBS; green, 0.1% FBS; red, 0% FBS). 2 ng/ml exogenous TGF-β1 shows little effect on cell density (open symbols and dotted lines) at any serum concentration. (B and C) Dashed lines indicate that cell density values are normalized to the global average obtained at 4 h. (D) Quantification of substrate stiffness effects on fibroblast apoptosis (blue indicates percentage of cells exhibiting caspase 3/7 activity after 24-h serum deprivation) and proliferation (red indicates percentage of nuclei positive for BrdU incorporation in 10% FBS). Standard deviation is from three independent experiments. (E) In panels B–D, colored triangles along the x axis are used to indicate the interquartile and median stiffness values of lung parenchyma from saline (cyan)- or bleomycin (red)-treated mice. (F) Fibroblast migration speed and persistence vary along substrate stiffness as measured with time-lapse video microscopy. Error bars indicate SD from 12 cells for each condition from two independent experiments. (G) Fibroblast migration tracks on stiffness gradient gels obtained from time-lapse video microscopy. Digital images were taken every 2 min for a total of 5 h per experiment. Each wind rose plot shows centroid tracks from 7–10 representative cells from each indicated stiffness region, with the initial position of each track superimposed at a common origin. Bars: (A) 500 µm; (G) 50 µm.
conditions spanning 100–25,600 Pa in fourfold increments. Cells grown from three independent human donors were used, and we focused our attention on genes with cross-donor rank correlation among stiffness conditions of >0.4, limiting analysis to the 2,462 probes (representing 1,829 unique genes) expressed in a consistent fashion among donors. We further restricted our attention to genes with a minimum within-donor coefficient of variation (of their exponentiated, base 2, robust multichip average [RMA] signal) across stiffness conditions of ≥0.2, resulting in a selection of 192 probes (150 of which have a gene assignment), representing 124 unique genes which we classified as responsive to alterations in stiffness across all three donors. Hierarchical clustering based on linear correlation across the three donors identified two major clusters of genes, those whose expression generally increased with stiffness (Fig. S4 A) and those that decreased (Fig. S4 C) with increasing stiffness. Ontological analysis of the cluster of genes increasing with stiffness identified highly significant enrichment for mitosis and microtubule motor activity (Fig. S4 B), which is consistent with our prior measurement of enhanced cell division across increasing matrix stiffness (Fig. 2 D).

Stiffness-dependent fibroblast gene expression
To more rigorously test the assertion that increasing stiffness promotes expression of a fibrogenic cellular program, we performed a microarray analysis of RNA harvested from normal human lung fibroblasts (NHLFs) grown on five discrete stiffness conditions spanning 100–25,600 Pa in fourfold increments. Cells grown from three independent human donors were used, and we focused our attention on genes with cross-donor rank correlation among stiffness conditions of >0.4, limiting analysis to the 2,462 probes (representing 1,829 unique genes) expressed in a consistent fashion among donors. We further restricted our attention to genes with a minimum within-donor coefficient of variation (of their exponentiated, base 2, robust multichip average [RMA] signal) across stiffness conditions of ≥0.2, resulting in a selection of 192 probes (150 of which have a gene assignment), representing 124 unique genes which we classified as responsive to alterations in stiffness across all three donors. Hierarchical clustering based on linear correlation across the three donors identified two major clusters of genes, those whose expression generally increased with stiffness (Fig. S4 A) and those that decreased (Fig. S4 C) with increasing stiffness. Ontological analysis of the cluster of genes increasing with stiffness identified highly significant enrichment for mitosis and microtubule motor activity (Fig. S4 B), which is consistent with our prior measurement of enhanced cell division across increasing matrix stiffness (Fig. 2 D). A similar analysis
Because fibroblasts interacting with matrices of increasing stiffness have been shown to alter activation of local matrix-incorporated TGF-β (Wipff et al., 2007), we sought to compare stiffness response genes to the transcriptional changes evoked by treatment with exogenous TGF-β1. We analyzed a previously published dataset reporting TGF-β1–responsive genes in NHLFs (Kapoun et al., 2006), identifying 1,176 unique genes responsive to this stimulus. Although there are subtle differences in culture conditions and passage number that should be noted in comparing our results with the TGF-β–responsive gene list, of 124 stiffness-responsive genes, only 33 genes were identified as also responsive to TGF-β1 (Fig. S4). Strikingly, among these 33 genes, expression levels of 18 were driven in opposite directions by the two stimuli (e.g., increasing with stiffness and decreasing with TGF-β1). This comparative analysis strongly suggests that the response to substrate stiffness identified in our microarray analysis is largely independent from a TGF-β–driven transcriptional program.

Matrix stiffness promotes fibrogenesis through COX-2 suppression

One intriguing candidate gene that emerged from the microarray analysis was PTGS2 (Fig. 4 C), which encodes the COX-2 enzyme. The COX enzymes convert arachidonic acid into prostaglandin H2, which is further processed into various prostaglandins, including PGE2. Notably, PGE2 is the most abundant of the subcluster of genes most prominently attenuated with increasing stiffness (Fig. 4 A) identified negative regulation of proliferation as a highly enriched ontology, as well as several functional annotations related to propeptide activation and proteolytic activity in the extracellular space (Fig. 4 B). These ontologies were conspicuous for their inclusion of gene members MMP3, MMP10, and cathepsin K (CTSK), all of which are proteolytic for matrix proteins, including gelatin and fibrillar collagens. CTSK in particular has been shown to play a key role in protecting against lung fibrosis (Bühling et al., 2004). The microarray analysis also confirmed the changes in COL1A1 and MMP1 noted previously (Fig. 3 C), although the changes were modest relative to the strongest signals emerging from the microarray analysis (Fig. 4 C). Analysis by quantitative PCR (qPCR) confirmed the changes in transcript levels for MMP10, MMP3, and CTSK, along with PTGS2 across stiffness conditions (Fig. 4 C). Collectively, these data suggest that matrix stiffening drives a broad gene expression program in support of fibroblast proliferation and selective but coordinated suppression of genes encoding collagen-degrading enzymes. Coupled with our demonstration that stiffness enhances procollagen synthesis and suppresses MMP1 expression (Figs. 3 and 4), our findings are consistent with a potentially devastating adverse feedback loop in which progressive matrix stiffening begets enhanced matrix deposition and decreased matrix degradation by fibroblasts, promoting self-sustaining fibrosis.
prostaglandin in the airways, is present at high concentrations in healthy lungs, and is the predominant prostaglandin product of alveolar epithelial cells and lung fibroblasts (Ozaki et al., 1987; Huang and Peters-Golden, 2008). We confirmed that increasing matrix stiffness dramatically attenuates PTGS2 expression by qPCR (>80% reduction; Fig. 4 C), and extended this to show greatly diminished autocrine levels of PGE2 present in the supernatants of cultured fibroblasts on matrices of increasing stiffness (Fig. 5 A). The PGE2 levels were largely dependent on COX-2 activity, as selective inhibition of COX-2 with NS-398 (IC50 for COX-2 is 1.8 µM and for COX-1 is 75 µM) reduced soluble PGE2 levels by 90–100% at low stiffness. We did not observe stiffness-dependent changes in expression of either COX-1 (PTGS1) or prostanoid receptor (PTGER1–4) levels (Fig. 4 C), further confirming that suppression of COX-2 expression was the dominant factor in the attenuation of PGE2 levels with increasing stiffness.

PGE2 has been shown to suppress fibroblast proliferation and collagen synthesis by signaling through the EP2 prostanoid receptor (Choung et al., 1998; Huang et al., 2007). We confirmed that suppression of PGE2 levels was a necessary step in stiffness-induced fibroblast accumulation by treating with exogenous PGE2, EP2 agonist (butaprost; Buta), and COX-2 inhibitor (NS-398), respectively. (E) Immunostaining for procollagen I in NHLFs (1% FBS). EP2 agonist (5 µM butaprost) abrogates the stiffness effect on procollagen I expression, whereas COX-2 selective inhibitor (5 µM NS-398) is unable to augment procollagen I expression at low stiffness. Bar, 50 µm. (F) Relative mRNA levels for COL1A1, assessed by qPCR, in A549 human lung epithelial cells, primary human dermal, synovial and lung (NHLF) fibroblasts, and airway smooth muscle (ASM) cells, as well as CCL-151 and IMR-90 lung fibroblast cell lines (0.1% FBS). (A–D and G) Colored triangles along the x axis are used to indicate the interquartile and median stiffness values of lung parenchyma from saline (cyan)- or bleomycin (red)-treated mice.

Figure 5. PGE2 regulates fibroblast responses to increasing stiffness. (A) Relative PGE2 levels in NHLF cell culture supernatants with and without selective COX-2 inhibitor (NS-398). (B–D) NHLF accumulation in discrete stiffness wells (1% FBS) and modification by exogenous PGE2, EP2 agonist (butaprost; Buta), and COX-2 inhibitor (NS-398), respectively. (E) Immunostaining for procollagen I in NHLFs (1% FBS). EP2 agonist (5 µM butaprost) abrogates the stiffness effect on procollagen I expression, whereas COX-2 selective inhibitor (5 µM NS-398) is unable to augment procollagen I expression at low stiffness. Bar, 50 µm. (F) Relative mRNA levels for COL1A1, assessed by qPCR, in A549 human lung epithelial cells, primary human dermal, synovial and lung (NHLF) fibroblasts, and airway smooth muscle (ASM) cells, as well as CCL-151 and IMR-90 lung fibroblast cell lines (0.1% FBS). (A–D and G) Colored triangles along the x axis are used to indicate the interquartile and median stiffness values of lung parenchyma from saline (cyan)- or bleomycin (red)-treated mice.
Figure 6. ROCK and PGE₂ exert mutually antagonistic effects on fibroblasts. (A) Increasing stiffness enhances organization of filamentous F-actin cytoskeleton (red) and triggers formation of paxillin-positive focal adhesions (green). The effect of stiffness on formation of these cellular structures is reversed with 30 ng/ml exogenous PGE₂, in a fashion identical to treatment with 33 µM of the ROCK inhibitor Y27632 (1% FBS). (B) Increasing stiffness drives expression and assembly of α-SMA (green) into F-actin stress fibers (red). The effect of stiffness on α-SMA is reversed with 30 ng/ml exogenous PGE₂, in a fashion identical to treatment with 33 µM of the ROCK inhibitor Y27632 (1% FBS). (C) 33 µM Y27632 increases expression of COX-2 (PTGS2) mRNA across matrix stiffness conditions (0.1% FBS), relative to untreated cells (CTRL). Error bars indicate SD from three replicate samples from one representative experiment. (D) 33 µM Y27632 equilibrates endogenous PGE₂ levels across matrix stiffness conditions (0.1% FBS), relative to untreated cells (CTRL). Error bars indicate SD from three independent experiments. (E) Results are consistent with mutually antagonistic and self-reinforcing
promoting fibroblast activation (Fig. 6 E). Because these fibroblast states lead to self-reinforcing effects on matrix synthesis and degradation (Figs. 2–4), matrix stiffening and the accompanying shift in balance between quiescence and activation represent a critical step toward self-sustaining fibrosis.

**Discussion**

With the use of AFM microindentation, we found that emerging fibrotic lesions in the murine model of bleomycin-induced fibrosis exhibit median stiffness sixfold higher than surrounding normal lung parenchyma. The increases in stiffness far exceed previous macroscale measurements of fibrotic stiffening of the lung (Ebihara et al., 2000), and their highly localized nature is consistent with the patchy nature of both the bleomycin model and human disease (Selman et al., 2001; Moore and Hogaboam, 2008). Although prior work has established that fibroblast proliferation and myofibroblast differentiation vary with matrix stiffness (Arora et al., 1999; Wang et al., 2000; Goftin et al., 2006; Li et al., 2007), our work shows for the first time that fibroblast proliferation and procollagen I synthesis are completely suppressed at the median stiffness of normal lung tissue, even in the presence of serum, suggesting a dominant role for normal lung compliance in maintaining the fibroblast quiescence typically observed in vivo but, notably, not in cell culture on rigid surfaces. Across the pathophysiological range identified in emerging fibrotic lesions, we observed that matrix stiffness progressively activates fibroblasts to proliferate and for the first time documented a coordinated program of enhanced collagen synthesis and suppression of transcripts encoding matrix-degrading proteases. Together, these findings establish that stiffness within the pathophysiological range exerts profound effects on the key aspects of fibroblast biology central to lung fibrosis. Based on these findings, we propose that an adverse feedback cycle of matrix stiffening, fibroblast proliferation, and net matrix synthesis underlies the relentless progressive nature of severe lung fibrosis.

Our study identifies suppression of COX-2 expression and PGE2 synthesis as necessary elements in the fibroblast proliferative and matrix synthetic responses to increasing matrix stiffness. Although various eicosanoids have been demonstrated to play both positive and negative roles in animal models of fibrosis (Lovgren et al., 2006; Oga et al., 2009), COX-2 and PGE2 levels are known to be reduced in the lungs of patients with fibrosis relative to healthy individuals (Borok et al., 1991; Xaubet et al., 2004), and fibroblasts isolated from fibrotic lungs are known to be deficient in PGE2 synthesis (Wilborn et al., 1995; Vancheri et al., 2000; Keerthisingam et al., 2001). Our data provide the first physiological explanation for these deficiencies. Exogenous PGE2 is well appreciated to suppress fibroblast proliferation and activation (Lama et al., 2002; Huang et al., 2007; Sandulache et al., 2007; Thomas et al., 2007; Weinberg et al., 2008).
2009) and limit experimental lung fibrosis (Failla et al., 2009), but our data expand on this by demonstrating that endogenous PGE$_2$ is secreted at high levels by fibroblasts growing on physiological stiffness matrices, and this capacity is gradually lost as matrix stiffness increases. However, notably, on soft substrates, pharmacological treatment to suppress COX-2 activity alone was insufficient to engage fibroblast proliferation and collagen synthesis, demonstrating that redundant mechanisms protect against spontaneous fibroblast activation when cells are resident in an environment as compliant as the normal lung. These findings are supported by animal models in which genetic deletion of COX-2 alone was insufficient to initiate fibrosis in the lung but did increase susceptibility to and severity of experimentally induced lung fibrosis (Keerthisingam et al., 2001; Bonner et al., 2002; Hodges et al., 2004). Collectively, our findings document a double layer of protection against fibrosis in the lung through the innate suppressive effects of the lung’s mechanical compliance, coupled with the abundant PGE$_2$ production it engenders.

TGF-β is widely acknowledged to play a key role in driving fibrogenesis through its effects on activation of fibroblasts to myofibroblasts and stimulation of matrix synthesis (Tomasek et al., 2002). Recent work indicates that fibroblasts grown for several days on matrices of varying stiffness activate matrix-bound TGF-β in a stiffness-dependent fashion (Wipff et al., 2007). We were surprised to find through microarray analysis that the early (48 h) fibroblast genomic response to stiffness is both supportive of proliferation and suppressive of collagen degradation but largely diverges from responses driven by TGF-β. Nowhere was this divergence more clear than in the case of COX-2. Although we found that increasing matrix stiffness suppressed COX-2 expression and downstream synthesis of PGE$_2$, TGF-β has been shown to stimulate COX-2 expression and PGE$_2$ production (Diaz et al., 1989; McAnulty et al., 1995; Keerthisingam et al., 2001; Kapoun et al., 2006). Because PGE$_2$ acts to suppress TGF-β–driven responses in fibroblasts (Kolodskick et al., 2003; Sandulache et al., 2007; Thomas et al., 2007), the COX-2/PGE$_2$ stimulation by TGF-β is thought to dampen cellular responses to this fibrogenic cytokine and prevent overexuberant fibrotic reactions. Our data raise the possibility that fibroblasts resident on stiff matrices experience enhanced responses to TGF-β because of stiffness-dependent down-regulation of a usual brake on TGF-β signaling. This may account, in part, for the emergence of TGF-β responses that we and others (Arora et al., 1999; Goffin et al., 2006) observe on matrices of increasing stiffness.

How variations in matrix stiffness are transduced by cells remains an area of intense investigation. Several important molecular players have emerged, including integrins (Paszek et al., 2005; Friedland et al., 2009), focal adhesion proteins such as FAK (Pelham and Wang, 1997; Paszek et al., 2005; Assoian and Klein, 2008), and cytoskeletal tension driven by the action of myosin II (Engler et al., 2006). Our results indicate that an antagonistic relationship between ROCK and COX-2 activities shapes fibroblast responses to matrix stiffness, wherein ROCK suppresses COX-2 expression and activity, whereas the COX-2 downstream product PGE$_2$ acts in a fashion similar to a ROCK inhibitor in disassembling the focal adhesions and stress fibers that form as matrix stiffness increases. This mutually antagonistic relationship sets up two feedback loops, both self-reinforcing, that drive fibroblasts to one of two extremes, activated (high Rho and low COX-2) or quiescent (low Rho and high COX-2). We speculate that in the lung, abundant PGE$_2$, produced by fibroblasts and surrounding epithelial cells (Ozaki et al., 1987; Wilborn et al., 1995; Lama et al., 2002), normally maintains the quiescent phenotype but that lung injury and matrix stiffening act in part through effects on PGE$_2$ levels to tilt the balance toward persistent, self-sustaining fibroblast activation (Fig. 6 D). Although we have not pinpointed the precise molecular interactions between Rho/ROCK and COX-2/PGE$_2$ in these experiments, PGE$_2$ is known to signal through cAMP in lung fibroblasts (Choung et al., 1998; Huang et al., 2007), and cAMP and downstream protein kinase A have been shown to both inactivate Rho and lead to cofilin-mediated actin depolymerization in multiple cell types (Dong et al., 1998; Lang et al., 1996; Murthy et al., 2003; Goeckeler and Wysolmerski, 2005), which is consistent with our observations.

How increasing matrix stiffness regulates expression of COX-2 is unknown. Recent findings indicate that diminished expression of COX-2 in fibroblasts isolated from fibrotic lungs is caused by defective histone acetylation in the COX-2 promoter (Coward et al., 2009). Epigenetic regulation of COX-2 is consistent with the observations that certain stimuli can provoke long-lasting changes in COX-2 expression that persist through multiple cell divisions (Korn, 1983) and could explain how deficiencies in PGE$_2$ secretion remain stable in serially passaged cells in culture (Wilborn et al., 1995; Vancheri et al., 2000; Keerthisingam et al., 2001). Although not yet well established, early evidence suggests that changes in matrix stiffness and cell shape do indeed regulate global levels of histone acetylation (Le Beyec et al., 2007). Further studies will be needed to elucidate the molecular links connecting matrix stiffness to gene expression and to test the role that epigenetic regulation plays in stiffness-dependent cellular behaviors.

In conclusion, we demonstrate for the first time that the median stiffness of normal lung tissue strongly inhibits the fibroblast functions at the core of fibrosis and elucidate a novel mechanism linking matrix stiffening to fibroblast activation in part through suppression of an endogenous COX-2/PGE$_2$ inhibitory pathway. Our data imply that fibrotic stiffening of a critical mass and extent, once established, is sufficient to generate a self-sustaining fibrotic process by virtue of its activation of fibroblasts, suppression of autoinhibitory COX-2/PGE$_2$, and subsequent feedback amplification of stiffness through net matrix synthesis. Importantly, our results imply that fibroblasts within a stiffened fibrotic lesion are primed to engage in further fibrogenic activities. The instability inherent in the presence of a mechanobiological profibrotic feedback loop could help to explain the variable course of the disease and rapid declines in lung function seen in exacerbations of lung fibrosis (Martinez et al., 2005; Collard et al., 2007). Based on the fact that similar stiffness levels and cellular behaviors are observed during fibrogenesis in other soft tissues (Georges et al., 2007; Li et al., 2007; Wells, 2008), we propose that the mechanobiological feedback loop detailed in this study may represent a common feature of progressive fibrosis. Improved recognition of how constraints are broken to
engage this feedback loop may prove critical for developing new prevention or early intervention strategies, whereas further elucidation of the mechanisms linking matrix stiffness to ongoing fibroblast activation may lead to new therapeutic opportunities for the treatment of progressive fibrosis.

**Materials and methods**

**Animals and bleomycin administration**

C57BL/6 mice (The National Cancer Institute Animal Production Program) were instilled intratracheally with 0.05 U bleomycin (Genex Sicot Pharmaceuticals Inc.) in 50 µl sterile saline or with 50 µl sterile saline for control mice, with lung tissue harvested 14 d later (Tager et al., 2008). All experiments were performed in accordance with National Institutes of Health guidelines and protocols approved by the Massachusetts General Hospital Subcommittee on Research Animal Care, and all mice were maintained in a specific pathogen-free environment certified by the American Association for Accreditation of Laboratory Animal Care.

**Lung parenchyma strip preparation and elastomapping**

Mouse lung parenchyma strips were prepared by infusing lungs with 2% low gel point agarose at 37°C (50 mL/kg body weight) followed by cooling to 4°C. The lung was excised, and slices of 5 cm thickness were cut with a razor blade from subpleural regions, excluding larger airways, and then washed in PBS at 37°C to remove the agarose (Bergner and Sanderson, 2003). The samples were placed on poly-l-lysine–coated coverslips. Samples were mechanically characterized using an atomic force microscope (MFP-3D; Asylum Research) by performing microindentation using a sphere-tipped probe (Novascan) with a diameter of 5 µm and a nominal spring constant of ~60 pN/nm. The cantilever spring constant was further confirmed by the thermal fluctuation method (Thundat et al., 1994). The AFM system was calibrated by following the manufacturer’s instruction before each indentation measurement. Force-indentation profiles were acquired at an indentation rate of 20 µm/s separated by 5 µm spatially in a 16 x 16 sample grid covering an 80 x 80-µm area. Shear modulus at each point on the grid was calculated from fitting force-indentation data using a Hertz sphere model (Richert et al., 2004), and resulting shear modulus data were plotted in contour maps. For lung tissue, a Poisson’s ratio of 0.4, 0.1, 1.6, 6.4, and 25.6 kPa. Cells were cultured with F12K containing 1% FBS at 37°C and 5% CO2. After 48 h, cell density was determined using CyQuant NF Cell Proliferation Assay (Invitrogen). Density values were normalized to the global mean value from 4-h attachment. For drug treatments, PGE2, butaprost, or NS-398 was added after 4-h initial attachment. In general, NIHFs or CCL-151 cells were cultured in 0.1% FBS for microarray and qPCR analysis to minimize differences in transcript levels caused by variations in proliferation and cell cycle progression. NIHFs were cultured in 1% FBS for proliferation, procollagen expression, PGE2, and collagen secretion analysis; CCL-151 cells grew more slowly and were cultured in 10% FBS for proliferation and procollagen expression analyses.

**Proliferation and apoptosis assays**

Fibroblasts were cultured on stiffness gradients for 4 d and then exposed to 50 µM BrdU for 24 h, fixed, permeabilized with 0.5% Triton X-100, treated with 4N hydrochloric acid for 15 min, and then neutralized with 0.1 M sodium borate, pH 8.5, for 20 min. Cells were then stained with anti-BrdU antibody (Alexa Fluor 546 mAb IgG2; Invitrogen) to identify replicating cells and with Hoechst 33342 to determine total cell number. For measurement of apoptosis, fibroblasts cultured on stiffness gradients for 4 d were serum-starved for 24 h and then stained with Image-iT Live green Caspase Detection Kit (Invitrogen) to detect caspase 3/7 activity.

**Migration assay**

Cell migration data were generated using a time-lapse videomicroscopy system consisting of an inverted microscope (Leica) with 10x phase contrast objective, charge-coupled device camera, and automated data acquisition software (Meta Morph 6.1). A temperature and CO2 control system was used for the maintenance of cell viability. Cells grown on stiffness gradients for 24 h were imaged every 2 min for 5 h. Cell x-centroids and displacements were determined using MetaMorph 6.1 cell tracking module. Only cells that did not divide, touch other cells, or leave the image field during the experiment were used for data analysis. Single cell speed was derived by dividing total cell path length by the total time of migration. Single cell persistence was derived from nonlinear least-squares regression using individual cell speed and mean-squared cell displacement as described previously (Harms et al., 2005).

**Discrete stiffness gel preparation**

Glass-bottom 96-well plates (Matrical) were treated with a 0.4% aqueous solution of gemmethoxypropyltrimethoxysilane (Acros Organics). Solutions containing 0.075% ammonium persulfate, 0.15% tetramethylethylenediamine, and variable ratios of acrylamide/bisacrylamide (Bio-Rad Laboratories), were delivered into the well plate. A 96-pin block with affixed, hydrophobic glass squares corresponding to the diameter of the wells was inserted, sandwiching the polymerization solutions between two glass surfaces. Gel thickness was controlled by placing 100-µm-thick spacers in the corner wells. After polymerization, the gel surface was derivatized with heterobifunctional cross-linker Sulfo-SANPAH (Thermo Fisher Scientific; Felham and Wong, 1997). Monomeric collagen (PureCol) diluted in PBS at 100 µg/ml was delivered to each well and incubated for 4 h at room temperature. The well plate was rinsed in PBS and UV-sterilized before cell seeding.

**Microarray analysis**

NIHFs from three independent donors were seeded at a density of 500 cells/mm2 in 96-well plates containing gels with five individual discrete stiffnesses: 0.1, 0.4, 1.6, 6.4, and 25.6 kPa. Cells were allowed to attach to the gels in serum-free F12K media for 4 h and incubated at 37°C and 5% CO2. Media was then replaced with F12K containing 0.1% FBS to minimize differences in transcript levels caused by variations in proliferation and cell cycle progression. After 48 h, cells were lysed with 50 µl RNA lysis buffer per well (QIAGEN). Duplicate stiffness wells were pooled, and RNA was isolated (RNeasy mini kit). cRNA was quantified with RiboGreen.
RNA analysis
Lung fibroblasts (CLC-L1), NHLFs, and IMR90, normal human dermal fibroblasts, human airway smooth muscle cells, A549 lung epithelial cells, and human synovial fibroblasts were seeded at a density of 50 cells/mm² in 96-well plates containing individual discrete stiffness gels. Synovial fibroblasts were prepared and cultured from discarded synovial tissues from rheumatoid arthritis patients (American College of Rheumatology criteria), obtained with approval of the Brigham and Women’s Hospital Institutional Review Board from synovectomy or joint replacement procedures (courtesy of Z. Gu and M. Brenner, Harvard Medical School and Brigham and Women’s Hospital, Boston, MA). All cells were allowed to attach to the gels in serum-free F12K media for 4 h and incubated at 37°C and 5% CO₂. Media was then replaced with F12K containing 0.1% FBS. After 48 h, cells were washed with 50 µl of RNA lysis buffer per well (Qiagen); replicate stiffness wells were pooled, and RNA was isolated (RNaseasy mini kit) and quantified with RiboGreen reagent. 50 ng of each sample was primed with oligo (dt) and reverse transcribed into cDNA using Taqman reverse transcription reagents (Applied Biosystems) at 25°C for 10 min, 37°C for 60 min, and 95°C for 5 min. Equivalent amounts of each cDNA sample were added to Power SYBR green PCR master mix (Applied Biosystems) containing 1 µM of each of forward and reverse primer (Table S1) for mRNA amplification. Primers were designed with qPrimerDepot (http://primerdepot.ncbi.nlm.nih.gov) and are intron overlapping. qPCR was performed by incubating at 95°C for 10 min to activate AmpliTaq Gold DNA polymerase (Applied Biosystems) and then cycling 40 times at 95°C for 15 s and 60°C for 1 min. Ct values within each experiment were normalized against GAPDH and hybridized to GeneChip Human Gene 1.0 ST arrays according to the standard protocol (Affymetrix). The resulting raw CEL file data were processed using standard RNA normalization into a numerical data matrix for subsequent mathematical analyses. These data are publicly available at the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE22011. Gene expression analysis was limited to 2,462 probes with cross-donor rank correlation among stiffness conditions of ≥0.4. The analysis was further restricted to 192 probes [150 of which have a gene assignment] representing 124 unique genes with minimum within-donor coefficient of variation [of their exponentiated, base 2, RMA signal] across stiffness conditions of ≥0.2. Hierarchical clustering was performed on the selected 150 probes based on linear correlation across the three donors. Before hierarchical clustering, the stiffness expression signal profile of each gene was standardized to mean zero, variance one within each donor. Ontological analysis was conducted using DAVID functional annotation clustering tool (http://david.abcc.ncifcrf.gov/summary.jsp).

Soluble collagen assay
NHLFs were seeded at a density of 50 cells/mm² in 96-well plates containing individual discrete stiffness gels. Cells were cultured with F12K containing 1% FBS at 37°C and 5% CO₂. After 48 h, cell culture media were collected, and the PGE₂ enzyme-linked immunosorbent assay was performed according to manufacturer’s instructions (Cayman Chemicals). PGE₂ concentrations were normalized to the corresponding cell numbers and then to the values at the lowest stiffness.

Statistical analysis
Results are expressed as means ± SD. Data were analyzed by nonparametric Mann-Whitney-Wilcoxon test or t test using the Prism statistical program (GraphPad Software, Inc.). P values <0.05 were considered significant. Error bars depict SD.

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