

TNF- α -stimulated fibroblasts secrete lumican to promote fibrocyte differentiation

Darrell Pilling^{a,1}, Varsha Vakil^b, Nehemiah Cox^a, and Richard H. Gomer^{a,b,1}

^aDepartment of Biology, Texas A&M University, College Station, TX 77843; and ^bDepartment of Biochemistry and Cell Biology, Rice University, Houston, TX 77005

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In healing wounds and fibrotic lesions, fibroblasts and monocyte-derived fibroblast-like cells called fibrocytes help to form scar tissue. Although fibrocytes promote collagen production by fibroblasts, little is known about signaling from fibroblasts to fibrocytes. In this report, we show that fibroblasts stimulated with the fibrocyte-secreted inflammatory signal tumor necrosis factor- α secrete the small leucine-rich proteoglycan lumican, and that lumican, but not the related proteoglycan decorin, promotes human fibrocyte differentiation. Lumican competes with the serum fibrocyte differentiation inhibitor serum amyloid P, but dominates over the fibroblast-secreted fibrocyte inhibitor Slit2. Lumican acts directly on monocytes, and unlike other factors that affect fibrocyte differentiation, lumican has no detectable effect on macrophage differentiation or polarization. $\alpha 2\beta 1$, $\alpha M\beta 2$, and $\alpha X\beta 2$ integrins are needed for lumican-induced fibrocyte differentiation. In lung tissue from pulmonary fibrosis patients with relatively normal lung function, lumican is present at low levels throughout the tissue, whereas patients with advanced disease have pronounced lumican expression in the fibrotic lesions. These data may explain why fibrocytes are increased in fibrotic tissues, suggest that the levels of lumican in tissues may have a significant effect on the decision of monocytes to differentiate into fibrocytes, and indicate that modulating lumican signaling may be useful as a therapeutic for fibrosis.

fibrocyte | lumican | fibrosis | inflammation | decorin

During wound healing, monocytes leave the circulation, enter the tissue, and differentiate into fibroblast-like cells called fibrocytes (1–6). Fibrocytes are also found in the scar tissue-like lesions associated with fibrotic diseases such as pulmonary fibrosis, congestive heart failure, cirrhosis of the liver, and nephrogenic systemic fibrosis (3, 7–11). Fibrocytes express markers of both hematopoietic cells (CD34, CD45, Fc γ R, LSP-1, MHC class II) and stromal cells (collagens, fibronectin, and matrix metalloproteases) (2, 3, 12–14). Fibrocytes also promote angiogenesis by secreting VEGF, bFGF, IL-8, and PDGF (15). A key question about fibrocyte differentiation and fibrosis is why fibrocytes are readily observed in fibrotic lesions, but are rarely observed in healthy tissues (3, 10, 16–19).

Fibrosis is a dynamic process involving many cells besides fibrocytes (20, 21). In fibrotic lesions, tissue-resident fibroblasts proliferate and produce excessive amounts of extracellular matrix (ECM) that distorts tissue architecture, leading to tissue destruction (21, 22). Fibrocytes secrete a variety of cytokines including IL-13, TGF- β , CTGF, and TNF- α that promote the proliferation, migration, and extracellular matrix production by the local fibroblasts (15, 23–26). Conversely, fibroblasts secrete a variety of factors that promote leukocyte entry, survival, and retention during inflammation (27–30). An intriguing possibility is that a runaway positive feedback loop involving unknown signals from fibrocyte-activated fibroblasts back to fibrocytes may lead to the persistence of fibrotic lesions.

In this report, we show that fibroblasts stimulated with TNF- α secrete the small leucine-rich proteoglycan lumican, and that lumican promotes fibrocyte differentiation. In addition, we show that in a mouse pulmonary fibrosis model as well as human patients with pulmonary fibrosis, there appears to be an increase in

lumican levels in the lungs, suggesting that pulmonary fibrosis may be in part due to elevated lumican levels. These data suggest that lumican may be one of the unknown signals from fibroblasts to fibrocytes that mediates part of a fibroblast-fibrocyte feedback loop that potentiates fibrosis.

Results

TNF- α -Stimulated Fibroblasts Promote Fibrocyte Differentiation. To test the hypothesis that activated fibroblasts might secrete soluble factors that promote fibrocyte differentiation, we added conditioned medium from human fibroblasts incubated with TNF- α (FCM+TNF- α) to human peripheral blood mononuclear cells (PBMC) in conditions where some of the monocytes in the PBMC would normally differentiate into fibrocytes. We used TNF- α as the stimulus, as other profibrotic cytokines such as IL-4, IL-13, and TGF- β act directly on monocytes to regulate fibrocyte differentiation (31). TNF- α is produced by monocytes, macrophages, and fibrocytes, serum TNF- α levels are increased in fibrosis patients, and TNF- α induces fibrosis in animal models (32–36).

In the absence of fibroblast-conditioned medium, we observed 160–1,500 fibrocytes per 10⁵ PBMC from the different donors, similar to what we and others have previously observed (12, 13, 37, 38). We and others have previously shown that ~1–2% of PBMC or 10–20% of monocytes can readily differentiate into fibrocytes (12, 13, 39). Because of this variability, for each donor, fibrocyte numbers were normalized to serum-free controls. For all donors,

Significance

Fibrosis is involved in 30–45% of deaths in the U.S. The disease may involve a runaway positive feedback loop between fibroblasts and monocyte-derived fibrocytes. The signals from fibrocytes to fibroblasts include inflammatory cytokines such as TNF- α , but the signals from TNF- α -stimulated fibroblasts to fibrocytes are unknown. We find that one of these signals is the protein lumican, and show that lumican levels are increased in fibrotic lesions. The identification of lumican as a signal in fibrotic tissues that promotes fibrocyte differentiation represents a major advance in our understanding of the regulation of the innate immune system, supports the positive feedback loop model of fibrosis, and indicates that lumican-inhibiting drugs may be beneficial in regulating fibrosis.

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Conflict of interest statement: D.P. and R.H.G. are inventors on patents for the use of SAP as a therapeutic for fibrosing diseases, and patents for the use of SAP-depleting materials to enhance wound healing. D.P. and R.H.G. are members of the Science Advisory Board of, and have stock options from, Promedior, a start-up company that is developing SAP as a therapeutic for fibrosing diseases, and receive a share of milestone payments made by Promedior to Rice University.

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¹To whom correspondence may be addressed. Email: rgomer@tamu.edu or dpilling@bio.tamu.edu.

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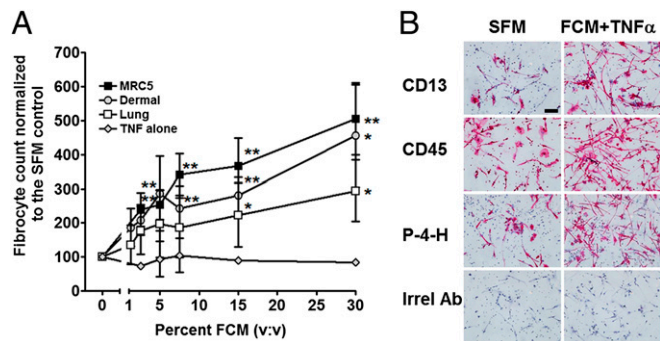


Fig. 1. TNF- α -stimulated fibroblasts secrete factors that potentiate fibrocyte differentiation. (A) Human normal adult lung, dermal, and MRC-5 fetal lung fibroblasts were incubated with 20 ng/mL TNF- α for 2 d, and the conditioned medium (FCM+TNF- α) was then collected. Human peripheral blood mononuclear cells (PBMC) were then cultured in the presence or absence of FCM+TNF- α for 5 d. As a control, PBMC were incubated with dilutions of SFM containing 20 ng/mL TNF- α . Values are mean \pm SEM ($n = 5$). * $P < 0.05$; ** $P < 0.01$ compared with the no-FCM control (t test). (B) PBMC were cultured for 5 d in SFM or SFM with 30% MRC-5 FCM+TNF- α . After 5 d, PBMC were air-dried, fixed, and stained with antibodies against the fibrocyte and macrophage marker CD13, the pan leukocyte marker CD45, prolyl-4-hydroxylase, a key enzyme in collagen synthesis, and mouse IgG1 antibodies (red staining). Cells were then counterstained with hematoxylin to identify nuclei (blue). Photomicrographs are representative results from five different donors. The spindle-shaped elongated cells are fibrocytes. (Scale bar: 20 μ m.)

compared with the control with no added FCM+TNF- α , 7.5% and above FCM+TNF- α led to a significant increase in fibrocyte differentiation (Fig. 1A). As previously observed (31), the presence of TNF- α alone did not significantly affect fibrocyte differentiation (Fig. 1A).

To verify that the FCM+TNF- α was affecting the number of fibrocytes, and to determine whether FCM+TNF- α altered the phenotype of fibrocytes, we stained PBMC after 5 d of culture with or without FCM+TNF- α for fibrocyte markers (Fig. 1B). In the absence of FCM+TNF- α , the elongated cells were positive for markers expressed by fibrocytes including CD13, CD45, and prolyl-4-hydroxylase (14). In the presence of 10% (vol/vol) FCM+TNF- α , the number of fibrocytes was significantly increased, although we did not detect any observable differences in the expression level per cell of CD13, CD45, and prolyl-4-hydroxylase (Fig. 1B).

Fibroblasts Secrete Lumican, which Promotes Fibrocyte Differentiation.

The factor (or factors) secreted by the fibroblasts were stable when stored for 2 wk at 4 $^{\circ}$ C or frozen at -20 $^{\circ}$ C or -80 $^{\circ}$ C. We tested the FCM+TNF- α for cytokines known to regulate fibrocyte differentiation or involvement in fibrotic responses. We did not detect any cytokines known to regulate fibrocyte differentiation, such as IL-4, IL-10, IL-12, IL-13, or IFN- γ (Fig. S1A). We did detect TNF- α and IL-6, but we have previously shown that these cytokines do not significantly affect fibrocyte differentiation (31). Fractionation with centrifugal filters indicated that the activity secreted by fibroblasts was a factor greater than 10 kDa but smaller than 100 kDa (Fig. S1B). Concentrates of FCM+TNF- α that passed through 100-kDa filters but were retained by 10-kDa filters were fractionated by ion exchange chromatography (Fig. S1C and D). The factor(s) eluted from an anion exchange column as a single peak between fractions 23 and 26 (Fig. S1C and D). Using mass spectrometry of tryptic digests from fraction 25, we identified several components of the active peak (Table S1). Of these, only albumin, the proteoglycan lumican, and the neuronal factor Slit2 are known to be extracellular proteins (40, 41). We previously observed that albumin does not regulate fibrocyte differentiation (37), and that Slit2 inhibits fibrocyte differentiation (41), suggesting that lumican may be the fibrocyte-inducing factor in FCM+TNF- α . To determine whether TNF- α increased lumican levels in human lung fibroblasts, cells were incubated in the presence or absence of TNF- α for 2 d. Compared with cells cultured in the absence of TNF- α , cells cultured in the presence of TNF- α had increased lumican staining and increased lumican levels in the conditioned medium (Fig. S2).

Lumican is a small leucine-rich proteoglycan related to fibromodulin, decorin, and biglycan (42). Lumican has a protein core of ~40 kDa, but is usually secreted as a higher molecular weight proteoglycan, with varying amounts keratan sulfate glycosaminoglycan (43, 44). Lumican is vital for the correct formation of collagen fibrils and promotes cell adhesion and migration (40, 45–47). Lumican knockout mice have multiple defects, including corneal opacity, skin and tendon fragility, and defective leukocyte migration (46, 48–51).

Recombinant human lumican, which is composed of the core 40-kDa protein and appears to be decorated with additional proteoglycan residues to give a mass of ~60 kDa (Fig. S3A), was added to PBMC to determine whether it can affect fibrocyte differentiation. Compared with PBMC cultured in SFM, cells cultured in the presence of lumican had significantly increased numbers of fibrocytes (Fig. 2A). The related SLRP decorin had

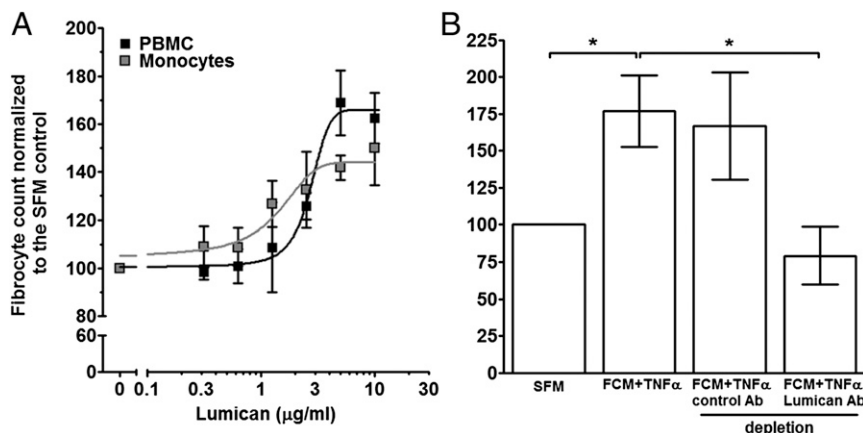


Fig. 2. Lumican potentiates human fibrocyte differentiation. (A) Human PBMC and isolated monocytes were cultured in the presence or absence of recombinant lumican for 5 d. Cells were then air-dried, fixed, stained, and fibrocytes were counted. Values are mean \pm SEM ($n = 3$). Lumican concentrations at 3 μ g/mL and above significantly inhibited fibrocyte differentiation (t test). Lines are fits to sigmoidal dose-response curves with variable Hill coefficients. (B) MRC-5 FCM+TNF- α was incubated with beads labeled with anti-lumican antibodies or goat IgG (a control IgG). Human PBMC were cultured in the presence or absence of 10% v:v antibody-depleted FCM+TNF- α for 5 d. Values are mean \pm SEM ($n = 4$). * $P < 0.05$ (one-way ANOVA, Tukey's test).

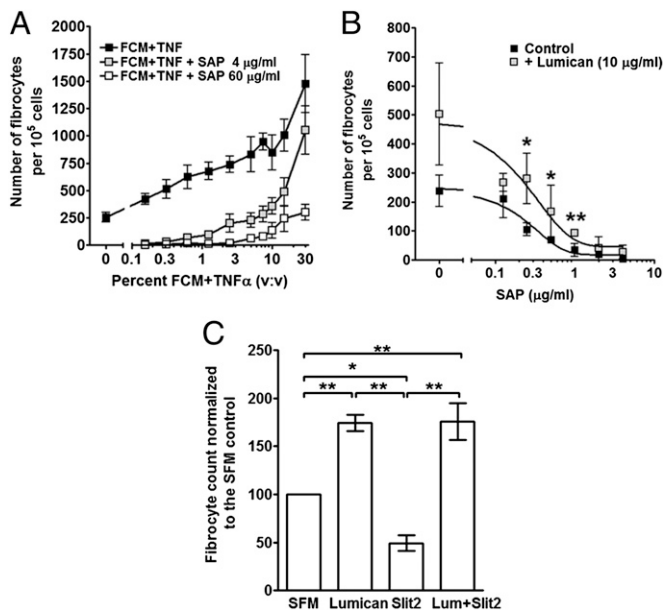


Fig. 3. The competition of lumican with SAP or Slit2. (A) PBMC were incubated with increasing concentrations of FCM+TNF- α in the presence of 0, 4, or 60 $\mu\text{g}/\text{mL}$ SAP. Values are mean \pm SEM ($n = 4$). (B) PBMC were incubated with increasing concentrations of SAP in the presence or absence of 10 $\mu\text{g}/\text{mL}$ lumican. After 5 d, cells were air-dried, fixed, and stained, and the number of fibrocytes was counted. Values are mean \pm SEM ($n = 3$). $**P < 0.01$ (t test). Lines are fits to sigmoidal dose-response curves with variable Hill coefficients. (C) PBMC were incubated in the presence or absence of lumican (10 $\mu\text{g}/\text{mL}$), Slit2 (500 pg/mL), or lumican and Slit2 combined. After 5 d, cells were air-dried, fixed, and stained, and the number of fibrocytes was counted. Values are mean \pm SEM ($n = 5$). $*P < 0.05$, $**P < 0.01$ (one-way ANOVA, Tukey's test).

no effect on fibrocyte differentiation (Fig. S3B). The lumican EC_{50} for promoting human fibrocyte differentiation was 2.9 ± 1.2 $\mu\text{g}/\text{mL}$, with a Hill coefficient of 7.4 ± 4.3 (mean \pm SEM, $n = 3$). To determine whether lumican altered the phenotype of fibrocytes, we stained PBMC after 5 d of culture with or without lumican for collagen-I. In the absence of lumican, $88.7 \pm 2.3\%$ (mean \pm SEM, $n = 3$) of fibrocytes were collagen-I positive, whereas in the presence of lumican $93.1 \pm 1.4\%$ were collagen-I positive, suggesting that lumican does not alter the expression of collagen-I. To determine whether the potentiation of fibrocyte differentiation by lumican is a direct effect on monocytes, or due to an indirect effect mediated by the B cells, dendritic cells, NK cells, or T cells present in the PBMC preparation, we incubated purified human monocytes with lumican (Fig. 2A). For all donors, lumican significantly promoted fibrocyte differentiation from isolated monocytes with an EC_{50} of 1.8 ± 0.9 $\mu\text{g}/\text{mL}$ and a Hill coefficient of 2.9 ± 0.9 (mean \pm SEM, $n = 3$). The EC_{50} and Hill coefficient for monocytes were not significantly different from those of PBMC (t tests). These data suggest that lumican acts directly on monocytes to potentiate fibrocyte differentiation.

To further test the hypothesis that lumican is a key factor in FCM+TNF- α that potentiates fibrocyte differentiation, lumican was immunodepleted from FCM+TNF- α . Compared with control IgG, immunodepletion with lumican antibodies reduced lumican levels by $\sim 82\%$ (Fig. S4). Immunodepletion with control IgG did not significantly alter the effect of fibrocyte differentiation by FCM+TNF- α (Fig. 2B). Compared with untreated FCM+TNF- α , immunodepletion of lumican from FCM+TNF- α led to a significant reduction in the ability of FCM+TNF- α to promote human fibrocyte differentiation (Fig. 2B). Together,

these data suggest that lumican is the active factor in FCM+TNF- α that inhibits fibrocyte differentiation.

To determine whether lumican alters the differentiation of monocytes, or the polarization of macrophages, PBMC were cultured for 6 d with or without lumican, or PBMC were differentiated into macrophages for 6 d, and then incubated for 3 d in the presence or absence of lumican. Cells were then stained with antibodies to the M1 markers CCR2, ICAM-1 (CD54), or CD86, or the M2 marker CD206 (Fig. S5). We did not detect any observable differences in the expression levels of these receptors, suggesting that lumican regulates monocyte to fibrocyte differentiation, rather than monocyte or macrophage polarization.

SAP Competes with Lumican to Regulate Fibrocyte Differentiation.

As fibrotic environments contain a wide variety of pro- and anti-fibrocyte inducing factors, we examined how SAP, a potent inhibitor of fibrocyte differentiation (13, 16, 38, 52), and FCM+TNF- α might compete to regulate fibrocyte differentiation. PBMC were cultured with increasing concentrations of FCM+TNF- α in the presence or absence of SAP. SAP inhibits human fibrocyte differentiation with an IC_{50} of ~ 0.3 $\mu\text{g}/\text{mL}$ (3 nM), and completely inhibits fibrocyte differentiation at 2 $\mu\text{g}/\text{mL}$ (13, 38, 53). In the presence of increasing concentrations of FCM+TNF- α we observed increased fibrocyte differentiation (Fig. 3A). When SAP was added to PBMC in SFM at either 4 $\mu\text{g}/\text{mL}$ (double the amount needed to inhibit fibrocyte differentiation (13), or 60 $\mu\text{g}/\text{mL}$ (double the average human plasma level of SAP; ref. 54), fibrocyte differentiation was reduced compared with cells cultured in FCM+TNF- α alone, but fibrocyte differentiation was not inhibited at the higher concentrations of FCM+TNF- α (Fig. 3A). These data suggest that the fibrocyte-potentiating factor in FCM+TNF- α competes with SAP to regulate fibrocyte differentiation.

To determine whether lumican also competes with SAP to regulate fibrocyte differentiation, PBMC were cultured in SFM with increasing concentrations of SAP in the absence or presence of 10 $\mu\text{g}/\text{mL}$ lumican (Fig. 3B). As observed previously (13, 38, 53), SAP inhibited fibrocyte differentiation with an IC_{50} of 0.29 ± 0.16 $\mu\text{g}/\text{mL}$. In the presence of lumican, the inhibitory activity of

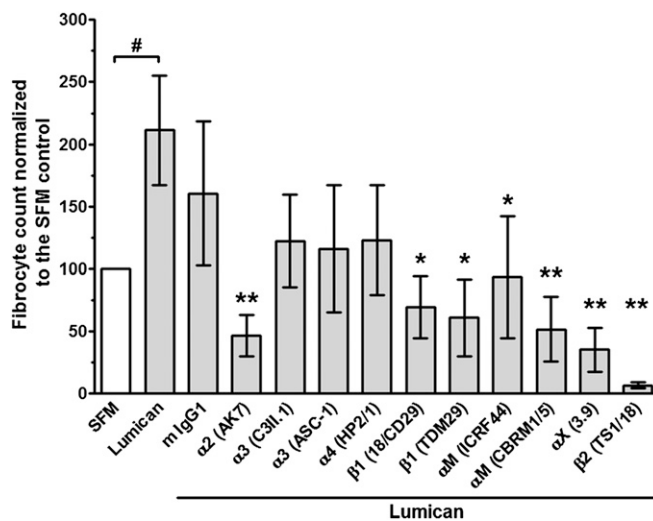


Fig. 4. Some anti-integrin antibodies inhibit lumican-induced fibrocyte differentiation. PBMC were incubated with 5 $\mu\text{g}/\text{mL}$ of mouse IgG1 or the indicated anti-integrin antibodies (specific integrin Ab in parenthesis), and then cultured in the presence or absence of 10 $\mu\text{g}/\text{mL}$ lumican. After 5 d, cells were air-dried, fixed, and stained, and the number of fibrocytes was counted. Values are mean \pm SEM ($n = 6$). # $P < 0.05$ compared with the SFM control (t test), $*P < 0.05$, $**P < 0.01$ compared with lumican control (one-way ANOVA, Dunnett's test).

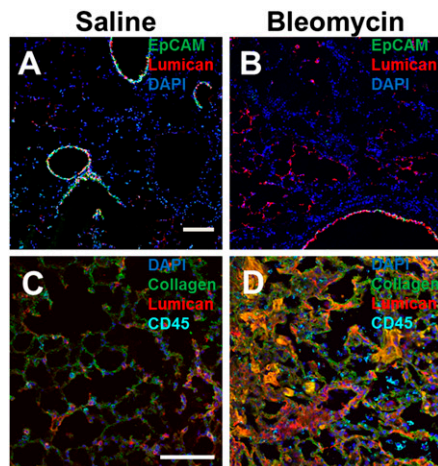


Fig. 5. Distribution of lumican in mouse lungs following bleomycin aspiration. Cryosections of mouse lungs 21 d after saline (A and C) or bleomycin (B and D) aspiration were incubated with antibodies against EpCAM (A and B, green) and rabbit anti-lumican (red), collagen (C and D, green), goat anti-lumican, (red), and CD45 (cyan). Nuclei were counterstained with DAPI (blue). (Scale bar: 100 μm .) Images are representative of three independent experiments.

SAP was reduced, with significantly more fibrocytes in cultures of SAP at 0.25, 0.5, and 1 $\mu\text{g}/\text{mL}$. Although the IC_{50} of SAP was shifted to $0.52 \pm 0.09 \mu\text{g}/\text{mL}$ in the presence of lumican, this was not significant (Fig. 3B; *t* test). These data indicate that lumican reduces the ability of SAP to inhibit fibrocyte differentiation.

Slit2 Does Not Inhibit Lumican-Induced Fibrocyte Differentiation. We previously found that fibroblasts secrete the neuronal guidance protein Slit2, and that Slit2 inhibits fibrocyte differentiation (41). To determine how lumican and Slit2 might compete to regulate fibrocyte differentiation, PBMC were cultured in SFM in the absence or presence of 10 $\mu\text{g}/\text{mL}$ lumican and 500 pg/mL Slit2. Slit2 inhibited fibrocyte differentiation, lumican potentiated fibrocyte differentiation, and the addition of Slit2 was unable to block this effect of lumican on fibrocyte differentiation (Fig. 3C). These data indicate that the fibrocyte-potentiating effect of lumican is dominant over the effect of Slit2.

Integrin-Blocking Antibodies Inhibit Lumican-Induced Fibrocyte Differentiation. Monocyte-derived fibrocytes express a wide variety of receptors that bind extracellular matrix proteins, including many $\beta 1$ and $\beta 2$ integrins (3, 4, 14). Lumican regulates fibroblast activation and migration via $\alpha 2\beta 1$ (CD49b/CD29) integrins (55, 56), and antibodies against αM (CD11b), $\beta 2$ (CD18), and $\beta 1$ (CD29) integrins inhibit neutrophil migration on lumican (57). To determine if these integrins are necessary for lumican potentiation of fibrocyte differentiation, we incubated PBMC with anti-integrin antibodies and then cultured the PBMC in the presence or absence of lumican. Antibodies to $\alpha 2$ (AK7), $\beta 1$ (18/CD29 and TDM29), αM (ICRF44 and CBRM1/5), αX (3.9), and $\beta 2$ (TS1/18) integrins inhibited lumican-induced fibrocyte differentiation, whereas antibodies to $\alpha 3$ (C3II.1 and ASC-1) and $\alpha 4$ (HP2/1) integrins had no significant effect (Fig. 4). As an alternative strategy to inhibit integrin-lumican binding, PBMC were incubated with the $\alpha 2$ integrin small molecule inhibitor BTT 3033 (58). At 500 nM, BTT3033 did not significantly regulate fibrocyte differentiation in the presence or absence of decorin, but did significantly inhibit lumican-induced fibrocyte differentiation (Fig. S6). Because $\alpha 2$ binds to $\beta 1$, and αM and αX bind to $\beta 2$ (59), these data suggest that $\alpha 2\beta 1$, $\alpha M\beta 2$, and $\alpha X\beta 2$ integrins are important for lumican potentiation of fibrocyte differentiation.

Lung Lumican Levels Increase in a Mouse Model of Pulmonary Fibrosis.

Lumican is present in human and murine lungs, and serum lumican levels are increased following lung inflammation and asthma (50, 60, 61). To determine whether lung lumican levels are up-regulated in pulmonary fibrosis, we stained lung tissue from mice that aspirated bleomycin or saline. At 21 d after saline exposure, lumican localized to alveolar walls and EpCAM-positive airway epithelial cells (Fig. 5). At 21 d, compared with mice that received saline, mice that aspirated bleomycin had more lumican staining, especially in the walls of alveoli (Fig. 5). In addition, the CD45-positive, collagen-I-positive cells were closely associated with areas of lumican (Fig. 5 and Fig. S7). These data suggest that in a mouse model, pulmonary fibrosis is associated with increased local accumulation of lumican in the lungs.

Lumican Levels Are Abnormally High in Human Pulmonary Fibrosis Lesions.

To determine if lumican is associated with human lung fibrosis, we examined the distribution of lumican in lung tissue from chronic obstructive pulmonary disease (COPD) patients with relatively normal lungs (>80% forced vital capacity; FVC) and pulmonary fibrosis patients with advanced disease (<50% FVC) (Table S2). Lung tissue from patients with an FVC of >80% showed limited lumican staining (Fig. 6A). In lung tissue

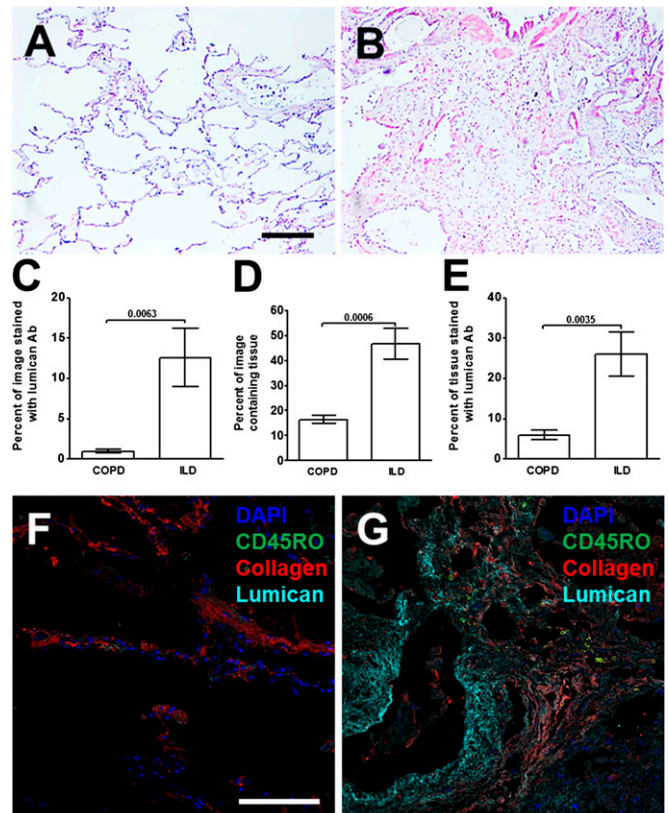


Fig. 6. Increased lumican in human lungs with pulmonary fibrosis. Lung tissue sections from COPD or pulmonary fibrosis patients were stained with anti-lumican antibodies. (A) Section from COPD patient with FVC >80%. (B) Section from IPF patient with FVC <50%. Bar is 0.2 mm. (C) The percentage area of the image stained by lumican antibodies. (D) The percentage of total area of image containing lung tissue. (E) The percentage of lung tissue stained by lumican antibodies. Values are mean \pm SEM, $n = 5-7$ patients per group. (*t* test). Lung sections from a COPD patient with FVC >80% (F) or an IPF patient with FVC <50% were incubated with antibodies against CD45RO (green), collagen (red), and lumican (cyan) (G). In F and G, nuclei were counterstained with DAPI (blue). (Scale bar: 0.1 mm.) Images are representative of three different patients.

from pulmonary fibrosis patients with advanced disease, lumican distribution was significantly increased, and was especially pronounced in areas adjacent to epithelial layers (Fig. 6B). The pulmonary fibrosis lungs had a greater area of tissue (and thus less airspace) and a greater area of lumican staining than the COPD lungs (Fig. 6C and D). Ratios of these two values indicated that the fibrotic lungs had a greater percentage of the tissue showing positive staining for lumican than the COPD lungs (Fig. 6E). As seen in the mouse model, compared with COPD patients, in the fibrotic lungs CD45RO-positive, collagen-I-positive cells were closely associated with areas of lumican (Fig. 6F and G and Fig. S8). These data suggest that human pulmonary fibrosis may involve an increase in the levels of lumican, and that these areas may promote fibrocyte differentiation.

Discussion

Tissue lumican levels are increased in cardiac and liver fibrosis (47, 62, 63), and we observed that lumican levels are increased in pulmonary fibrosis. Fibrocytes are rare in normal heart, lung, and liver, whereas fibrocytes are readily detected in fibrotic lesions in these tissues (3, 10, 16–19). Fibrocytes play a key role in wound healing and fibrosis, and in agreement with our observation that lumican promotes fibrocyte differentiation, lumican knockout mice have poor wound healing and are resistant to carbon tetrachloride-induced liver fibrosis (3, 47, 64). Poor wound healing and reduced fibrosis of lumican knockout mice are thought to be related to reduced collagen tensile strength and disorganized collagen fibrils, suggesting that lumican promotes extracellular matrix stiffness (46, 65). In fibrotic lesions, the extracellular matrix has increased stiffness, leading to enhanced fibroblast proliferation, myofibroblast formation, and increased extracellular matrix deposition (66, 67). Combined, these observations suggest that elevated lumican, by increasing tissue stiffness, may promote fibrosis.

Fibroblasts bind to lumican using $\beta 1$ integrins, whereas hematopoietic cells use $\beta 2$ integrins (55–57, 68). Monocytes, macrophages, and monocyte-derived fibrocytes express both $\beta 1$ and $\beta 2$ integrins (14), and our results indicate that both $\beta 1$ and $\beta 2$ integrins are involved in lumican-induced fibrocyte differentiation. The Hill coefficient of 7.4 ± 4.3 (mean \pm SEM) in the lumican dose–response curves indicates a cooperativity in lumican binding or the lumican signal transduction pathway. The ability of $\alpha 2$ integrin blocking antibodies and the small molecule inhibitor BTT3033, to inhibit lumican-induced fibrocyte differentiation mirrors the inhibition of cell migration observed with fibroblasts and melanoma cells (55, 56), and the ability of αM and αX antibodies to inhibit lumican-induced fibrocyte differentiation mirrors the inhibition of cell migration observed for leukocytes (57).

The ECM proteins collagen-IV, fibronectin, and vitronectin, which bind to integrin receptors (59), have no significant effect on fibrocyte differentiation (69). However, collagen-I has a modest inhibitory effect on fibrocyte differentiation (69). As both collagen-I and lumican bind to $\alpha 2\beta 1$ integrin (70, 71), additional receptors, such as $\alpha M\beta 2$ and $\alpha X\beta 2$, may differentially regulate the effects of lumican and collagen-I on fibrocyte differentiation.

We detected slit2 in the conditioned media from TNF- α -stimulated fibroblasts, and observed that the fibrocyte-potentiating activity of lumican appears to dominate over the fibrocyte-inhibiting activity of slit2. These data suggest a model where fibroblasts constitutively secrete slit2 to inhibit fibrocyte differentiation, but when there is tissue damage, inflammatory signals induce fibroblasts to up-regulate lumican production to drive fibrocyte differentiation. That the inflammatory signals use fibroblasts as an intermediate, rather than signaling directly to the fibrocytes, suggests that additional information obtained by fibroblasts might allow fibroblasts to modulate the lumican signal to fibrocytes. The observations that the fibrocyte potentiating signal from TNF- α -stimulated fibroblasts can be removed with anti-lumican antibodies, that high concentrations of SAP can strongly inhibit fibrocyte differentiation in the presence of high concentrations of lumican, and that high concentrations of conditioned media from TNF- α -stimulated fibroblasts can potentiate fibrocyte differentiation in the presence of high concentrations of SAP suggest that stimulated fibroblasts secrete factors that act in conjunction with lumican to counteract the effect of SAP. Together, our results support a model where a positive feedback loop between fibroblasts and fibrocytes, involving multiple signals, potentiates fibrosis. Lumican appears to be a major signal in this loop, suggesting that lumican-inhibiting drugs may be beneficial in regulating fibrosis.

Materials and Methods

All animals were used in accordance with National Institutes of Health guidelines and with a protocol approved by the Texas A&M University Institutional Animal Care and Use Committee. Human blood was obtained with written consent and with specific approval from the Texas A&M University human subjects Institutional Review Board. Human PBMC, monocytes, and fibroblasts were isolated, cultured, and incubated with antibodies, as described (37, 41). Fibrocytes were identified as described (13, 37). Fibroblast conditioned medium was fractionated, and the immunodepletion of lumican was performed, as described (13, 41). Pulmonary fibrosis in mice was induced by bleomycin instillation, as described previously (52, 72, 73). Lung sections were prepared, fixed, and stained, as described (16, 52). Detailed information about mice, experimental procedures, and statistical analyses can be found in *SI Materials and Methods*.

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