Mechanosensing by the α6-integrin confers an invasive fibroblast phenotype and mediates lung fibrosis

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Matrix stiffening is a prominent feature of pulmonary fibrosis. In this study, we demonstrate that matrix stiffness regulates the ability of fibrotic lung myofibroblasts to invade the basement membrane (BM). We identify α6-integrin as a mechanosensing integrin subunit that mediates matrix stiffness-regulated myofibroblast invasion. Increasing α6-expression, specifically the B isoform (α6B), couples β1-integrin to mediate MMP-2-dependent pericellular proteolysis of BM collagen IV, leading to myofibroblast invasion. Human idiopathic pulmonary fibrosis lung myofibroblasts express high levels of α6-integrin in vitro and in vivo. Genetic ablation of α6 in collagen-expressing mesenchymal cells or pharmacological blockade of matrix stiffness-regulated α6-expression protects mice against bleomycin injury-induced experimental lung fibrosis. These findings suggest that α6-integrin is a matrix stiffness-regulated mechanosensitive molecule which confers an invasive fibroblast phenotype and mediates experimental lung fibrosis. Targeting this mechanosensing α6(β1)-integrin offers a novel anti-fibrotic strategy against lung fibrosis.
Matrix stiffening is a prominent feature of pulmonary fibrosis. Accumulating evidence suggests that mechanointeractions between fibrotic lung fibroblasts (known as myofibroblasts) and stiffened, fibrotic extracellular matrix (ECM) provide a feed-forward mechanism that amplifies lung fibrosis. Elucidating the key mechanosensitive molecules that confer fibrogenic properties to myofibroblasts may uncover novel therapeutic strategies for fibrotic lung diseases, including idiopathic pulmonary fibrosis (IPF).

Fibroblasts sense the mechanical properties of the ECM by integrin and non-integrin mechanoreceptors. Integrins are cell-surface heterodimers composed of non-covalently associated α- and β-transmembrane subunits. As the major force-bearing molecular links between cells and the ECM, integrins play a central role in determining how cells sense and respond to their mechanical environment. Containing integrins serve as cellular receptors for the members of laminin family, a major structural component of the basement membrane (BM). Integrin subunit β4 (ITGA6) dimerizes with either α1 (ITGB1) or α2 (ITGB4) integrin subunit to form β4α1 or β4α2-integrin complex. In normal human embryonic and adult tissues, β4-integrins are found to be prominently expressed in epithelia, whereas normal fibroblasts express little β4 integrins. β4 integrin is the main component of hemidesmosome and is expressed by airway epithelial cells in healthy adults. Mice null for β4-integrins are deficient in hemidesmosome formation and die at birth with severe blistering of skin and other epithelia. Recent studies showed that β4-integrin marks a subpopulation of lung epithelial progenitor cells which are capable of self-renewal and differentiation into multiple respiratory epithelial cell types. Although the role of β4-integrins in fibroblasts are limited, increasing β4-integrin expression has been observed in transformed neoplastic fibroblasts in human metastatic fibrosarcoma.

The BM is a dense, sheet-like structure at the interface of epithelial/endothelial and mesenchymal tissues. It maintains the polarity of lung epithelial cells and provides a physical barrier between lung epithelium and the mesenchyme. Cells invade the BM by adhering to BM matrices and engaging proteinase-dependent dissolution of BM at focal adhesions. Alternatively, cells may transmigrate through the BM by proteinase-independent disassembly of BM superstructure and enlargement of pore size, a process termed mesenchymal-amoeboid transition.

The integrity of the BM maintains a healthy lung epithelium and its integrity is essential for restoration of alveolar epithelial homeostasis following lung injury. Loss of the BM integrity has been observed in IPF. Mechanisms underlying disruption of the BM integrity in IPF are currently not well understood. Disruption of alveolar BMs prevents an orderly repair of the damaged alveolar type I epithelial cells, thus impairing normal reepithelialization. It has been observed that intact BMs suppress programmed cell death in mammary epithelium and other tissues, suggesting that loss of the BM integrity may also promote alveolar epithelial cell apoptosis.

Fibrotic lung fibroblasts are characterized by an invasive phenotype. reported that constitutively lower levels of PTEN in IPF lung myofibroblasts promote cell invasion into the BM, whereas ligation-dependent expression of PTEN prevents cell invasion. found that mouse lung myofibroblasts isolated from hyaluronan synthase 2 transgenic mice acquire an invasive phenotype. In a recent study, showed that knockdown of β-arrestin2 in IPF lung myofibroblasts attenuates cell invasiveness. Proteinases capable of degrading the BM include MMP2 and MMP9 of type IV collagenases. In the fibroblastic foci of IPF, subepithelial myofibroblasts close to the areas of BM disruption express MMP-2 as well as MMP-9 (ref. 25), suggesting that MMPs may mediate proteinase-dependent IPF myofibroblast invasion into the BM and the disruption of BM integrity.

In this study, we report that β4 is a matrix stiffness-regulated mechanosensitive integrin subunit. Stiff matrix-induced upregulation of β4-expression mediates IPF lung myofibroblast invasion into the BM. We explore mechanotransductive mechanisms for β4-integrin expression and demonstrate that the expression of this integrin subunit by lung myofibroblasts is increased in both human IPF and bleomycin injury-induced experimental lung fibrosis. Animal studies using genetic and pharmacological approaches support targeting mechanosensitive β4-integrin as a novel therapeutic strategy for lung fibrosis.

Results

β4 is a matrix stiffness-regulated mechanosensitive gene. To determine whether matrix stiffness regulates the expression of cell adhesion and ECM molecules, we performed a qPCR array analysis that contains 84 genes, including 16 integrin subunits in primary lung myofibroblasts isolated from patients with IPF (Supplementary Fig. 1). We found that 10 genes were increased or decreased ≥ twofold under stiff versus soft matrix conditions; the differential mRNA expression of 7 genes was statistically significant (Table 1). The β4-integrin subunit mRNA was increased 5.3-fold on stiff matrix. To validate these gene expression data, we performed additional studies at the protein level to determine if the β4-subunit is regulated by matrix stiffness; we observed a matrix stiffness grade-dependent increase in β4-integrin expression when cells were grown on polyacrylamide (PA) gels with stiffness ranging from 1 to 20 kPa (Fig. 1a). Similar results were obtained when cells were grown on a second stiffness-tunable substrate system of polydimethylsiloxane hydrogels (Supplementary Fig. 2a). Lung myofibroblasts isolated from bleomycin-treated mice also respond to matrix stiffening with increased β4-expression (Supplementary Fig. 2b,c). These results identify, for the first time, the β4-integrin subunit as a matrix stiffness-regulated mechanosensitive gene/protein.

A bioinformatics search identified AP-1-specific TPA-response elements (TREs) in the promoter region of human and mouse β4-integrin genes (Supplementary Fig. 2d). It has been shown that mechanical stretch activates AP-1 in human osteoblastic cells. Activation of AP-1 transcription complex is associated with cancer cell invasion. On the basis of this information, we sought to determine whether the AP-1 transcription complex mediates stiff matrix-induced β4-integrin

Table 1 | Matrix stiffness-regulated cell adhesion and ECM molecules.

| Gene     | Well  | Fold up or downregulation |
|----------|-------|--------------------------|
| MMP9     | F04   | 6.4 ± 1.6**              |
| ITGA6    | C10   | 5.3 ± 1.1**              |
| CTGF     | B08   | 4.7 ± 1.3**              |
| MMP12    | E07   | 3.0 ± 1.8*               |
| SPP1     | G01   | 2.4 ± 0.8*               |
| MMP16    | E11   | 2.1 ± 1.1                |
| MMP17    | E06   | – 5.1 ± 2.1**            |
| VCAM1    | G11   | – 2.6 ± 0.6**            |
| ADAMTS8  | A03   | – 2.3 ± 0.8**            |
| CLEC3B   | G09   | – 2.0 ± 1.2              |

Table 1. Matrix stiffness-regulated cell adhesion and ECM molecules.

Gene Well Fold up or downregulation Stiff/soft

| MMP9 | F04 | 6.4 ± 1.6** |
| ITGA6 | C10 | 5.3 ± 1.1** |
| CTGF | B08 | 4.7 ± 1.3** |
| MMP12 | E07 | 3.0 ± 1.8* |
| SPP1 | G01 | 2.4 ± 0.8* |
| MMP16 | E11 | 2.1 ± 1.1 |
| MMP17 | E06 | – 5.1 ± 2.1** |
| VCAM1 | G11 | – 2.6 ± 0.6** |
| ADAMTS8 | A03 | – 2.3 ± 0.8** |
| CLEC3B | G09 | – 2.0 ± 1.2 |

*Results are the means ± s.d. of three separate experiments; **P<0.05; ***P<0.01.

ECM, extracellular matrix.
gene expression. We first determined whether matrix stiffness regulates the promoter activity of α6-gene. A 6,200-bp of wild-type (WT) human proximal α6-promoter reporter and 3 mutated promoter reporters harbouring mutations at the specific AP-1-binding DNA sequences, TRE1 (−4,848 to −4,854 nt), TRE2 (−2,873 to −2,879 nt) or both TRE1 and TRE2, were transfected into IPF lung myofibroblasts (Fig. 1b). In cells transfected with WT α6-promoter reporter, stiff matrix significantly increased luciferase expression (Fig. 1b), suggesting that human α6-promoter activity is enhanced by stiff matrix. Deletion of either TRE1 or TRE2 inhibited stiff matrix-induced increases in α6-promoter activity. Deletion of both TRE1 and TRE2 completely blocked stiff matrix-induced α6-promoter activation (Fig. 1b). Altogether, these data suggest that stiff matrix activates α6-promoter by an AP-1-dependent mechanism. 

Next, we investigated effects of matrix stiffness on the binding of seven major AP-1 components (c-Fos, c-Jun, FosB, Fra1, Fra2, JunB and JunD) to immobilized TRES. Stiff matrix selectively increased c-Fos and c-Jun binding to immobilized oligonucleotides containing TRES, whereas the binding of FosB, Fra1, JunB and JunD to TRES were not altered by matrix stiffness (Fig. 1c). Previous studies have shown that Fos-related protein Fra2 is associated with human IPF and spontaneous development of lung fibrosis in mice. In our studies, neither the binding of Fra2 to immobilized TRES nor its expression or cytoplasmic/nuclear distribution were regulated by matrix stiffness (Fig. 1d), suggesting that matrix stiffness is unlikely to regulate Fra2 activity. It has been shown that phosphorylation of c-Fos at Ser32/Thr232 and c-Jun at Ser63/Ser73 is associated with increased DNA-binding activity of c-Fos and c-Jun. We found...
that stiff matrix (20 kPa) in comparison with soft matrix (1 kPa) increased the levels of phospho c-Fos at Ser32 and phospho c-Jun at Ser73 (Fig. 1e); the total protein and the mRNA levels of c-Fos and c-Jun were not altered by matrix stiffness (Fig. 1e and Supplementary Fig. 2f). Previously, we have shown that stiff matrix activates protein serine/threonine kinase ROCK in lung myofibroblasts5. Here, we observed that inhibition of ROCK by fasudil or siRNA-based knockdown blocked stiff matrix-induced c-Fos and c-Jun phosphorylation (Fig. 1f), suggesting that ROCK mediates stiff matrix-induced phosphorylation and activation of c-Fos/c-Jun-transcription complex. Quantitative chromatin immunoprecipitation assay demonstrated that stiff matrix significantly increased the constitutive enrichment of αcR-promoter DNA in phospho c-Fos antibody-immunoprecipitated chromatin of IPF lung myofibroblasts at both the proximal (−2,873/−2,879 nt) and distal (−4,848/−4,854 nt) TRE sites (Fig. 1g). Altogether, these data suggest that the c-Fos/c-Jun complex of AP-1 transcription factor family mediates matrix-dependent transactivation of αcR-gene.

To determine whether inhibition of c-Fos/c-Jun-dependent αcR-promoter activation blocks stiff matrix-induced αcR-expression, we used CRISPR interference (CRISPRi) technology, which allows sequence-specific disruption of transcription factor binding to the promoter for gene silencing31. Two single guide RNAs (sgRNAs) were designed to specifically bind to a 20-bp DNA sequence next to each of two TREs in human αcR-promoter (Fig. 1h). Expression of deactivated Cas9 (dCas9)-KRAB fusion proteins, in which dCas9 provides a DNA-binding platform at sites defined by sgRNAs for KRAB domain-mediated repression of c-Fos/c-Jun-dependent αcR-promoter activation, blocked stiff matrix-induced αcR-expression (Fig. 1h). Similar to CRISPRi, pharmacologic inhibition of c-Fos/c-Jun activity by T-5224, a selective c-Fos/AP-1 inhibitor, or by c-Jun peptide inhibitor also blocked stiff matrix-induced αcR-expression (Fig. 1i).

We also observed that stiff matrix increases c-fos and c-jun binding to immobilized TREs in mouse lung myofibroblasts, whereas the binding of fosB, fra1, fra2, junB and junD to TREs were not altered by matrix stiffness (Supplementary Fig. 2f). Quantitative chromatin immunoprecipitation assay demonstrated that stiff matrix significantly increased the enrichment of mouse αcR-promoter DNA in phospho c-Fos antibody-immunoprecipitated chromatin of mouse lung myofibroblasts (Supplementary Fig. 2g). Pharmacologic inhibition of c-Fos/c-Jun activity by T-5224 or decoy oligodeoxynucleotides32 blocked stiff matrix-induced mouse αcR-expression (Supplementary Fig. 2h). Taken together, these data support a role for the c-Fos/c-Jun-dependent mechanotransduction pathway in stiff matrix-induced αcR-expression.

αcR Mediates lung myofibroblast invasion. Fibrotic lung myofibroblasts isolated from patients with IPF are characterized by an invasive phenotype25–24. To determine whether the mechanical properties of the ECM may regulate the ability of IPF lung myofibroblasts to invade the BM, we pre-cultured primary lung myofibroblasts isolated from patients with IPF on soft (1 kPa) and stiff (20 kPa) PA hydrogel substrates. The stiffness grades of soft and stiff PA gels were within the physiologic stiffness ranges of normal and fibrotic lungs12. Lung myofibroblasts adapted to soft and stiff matrix were trypsinized and transferred to the invasion chambers containing BM matrices (Matrigel). We observed that cells derived from stiff PA gels had a significantly higher invasion index than cells derived from soft PA gels (Fig. 2a). Similar findings were observed when cells were cultured on soft (2 kPa) and stiff (30 kPa) polydimethylsiloxane hydrogels (Supplementary Fig. 3a). These data suggest that stiff matrix promotes IPF lung myofibroblasts to invade the BM. To confirm these findings, we designed a ‘sandwich’ invasion assay in which cells cultured on soft and stiff PA gels were directly transferred to invasion chambers with the apical (dorsal) side of cells in close contact with the BM matrices (Supplementary Fig. 3b). We observed enhanced invasive properties of lung myofibroblasts on stiff matrices using this second approach (Supplementary Fig. 3c). Since Matrigel may not fully replicate BM matrices found in vivo, we isolated rat mesenteric BM that has been used to study cancer cell invasion33 to determine effects of matrix stiffness on the ability of IPF lung myofibroblasts to invade biological BMs (Supplementary Fig. 3d). We observed that IPF lung myofibroblasts pre-cultured on stiff PA gels had a higher invasive index than cells pre-cultured on soft PA gels (Supplementary Fig. 3e). Altogether, these findings indicate that matrix stiffness confers an invasive property to IPF lung myofibroblasts, specifically through the BM.

αcR-integrin is a major cellular receptor for laminin, a protein component of the BM. Next, we determined whether the mechanosensing αcR-integrin regulates stiff matrix-induced lung myofibroblast invasion into the BM. We first compared the levels of αcR-integrin on the cell surface in the subpopulation of IPF lung myofibroblasts, that is, myofibroblasts that penetrated into the BM in comparison with the total population of IPF lung myofibroblasts. Flow cytometry analysis demonstrated a higher expression of αcR on the cell surface of invading lung myofibroblasts relative to the total lung myofibroblast population (Fig. 2b). When lung myofibroblasts were pre-treated with NKI-GoH3 (a specific antibody that blocks αcR-mediated cell adhesion) or T-5224 (an inhibitor of c-Fos), stiff matrix-dependent lung myofibroblast invasion into the BM was significantly inhibited (Fig. 2c). In these experiments, flow cytometry analysis demonstrated that treatment with T-5224 and GoH3 inhibited αcR-integrin on the cell surface (Fig. 2c). Vehicle controls (IgG isotype control antibody for GoH3; polyvinylpyrrolidone (PVP) for T-5224) had no effects on lung myofibroblast invasion and αcR-expression on the cell surface. To further determine the role of αcR-integrin in matrix stiffness-regulated lung myofibroblast invasion into the BM, we generated lung myofibroblasts that overexpress αcR-GFP fusion proteins or GFP alone with a lentiviral vector-based approach; an siRNA-based approach was utilized to generate lung myofibroblasts deficient in αcR-integrin expression (Fig. 2d). Overexpression of αcR significantly enhanced stiff matrix-induced IPF lung myofibroblast invasion into the BM, whereas knockdown of αcR significantly inhibited lung myofibroblast invasion (Fig. 2e). In addition, overexpression of αcR was sufficient to induce BM invasion of lung myofibroblasts cultured on soft matrices. GFP control lentiviruses and control siRNA had no effects on matrix stiffness-regulated myofibroblast invasion into the BM (Fig. 2e).

Altogether, these loss- and gain-of-function studies support a key role for the mechanosensitive αcR-integrin in mediating matrix stiffness-regulated IPF lung myofibroblast invasion into the BM. Proteolytic degradation of the BM proteins is critical for cellular invasion into the BM34. Next, we determined whether the αcR-integrin mediates proteolysis of collagen IV, a major component of the BM, using fluorescent dye-quenched (DQ)-collagen IV which is quenched in its native form and emits strong fluorescence on proteolytic hydrolysis35. Confocal immunofluorescent microscopy showed that IPF lung myofibroblasts derived from stiff matrix expressed αcR-integrin subunit; fluorescent signals from DQ-collagen IV were observed in the periphery of αcR-positive lung myofibroblasts, indicative of pericellular proteolysis of collagen IV in the BM (Fig. 2f). Blocking αcR-mediated cell adhesion with NKI-GoH3 antibody...
(Fig. 2g), inhibition of mecano-induction of \( \alpha_6 \)-expression by T-5224 (Fig. 2h), and knockdown of \( \alpha_6 \)-expression with \( \alpha_6 \)-specific siRNA (Fig. 2i) inhibited pericellular proteolysis of collagen IV. Overexpression of \( \alpha_6 \)-integrin enhanced pericellular proteolysis of collagen IV (Fig. 2j). The IgG isotype control antibody, PVP and scrambled control siRNA had no effects on proteolysis of collagen IV (Supplementary Fig. 4).

The matrix metalloproteinases (MMPs), MMP-2 and MMP-9, are known to degrade collagen IV\(^{35}\). In this study, we observed that stiff matrix induced an average of sixfold increases in MMP-9 mRNA as compared with soft matrix (Table 1), whereas MMP-2 mRNA expression was not altered by matrix stiffness. However, a direct comparison of relative mRNA expression in myofibroblasts cultured on soft and stiff matrix, whereas MMP-9 activities were undetectable (Supplementary Fig. 5b). Consistent with the qPCR findings, MMP-2 activities were not altered by matrix stiffness. These data suggest that IPF lung myofibroblasts primarily express MMP-2 of type IV collagenases. Next, we investigated whether MMP-2 is involved in \( \alpha_6 \)-mediated collagen IV degradation. Knockdown of MMP-2 by siRNA blocked pericellular proteolysis of DQ-collagen IV (Supplementary Fig. 5c) and IPF lung myofibroblast invasion into BM matrices (Supplementary Fig. 5d). Collectively, these data suggest that \( \alpha_6 \)-dependent invasion of IPF lung myofibroblasts requires pericellular proteolysis of BM collagen IV by MMP-2.

**\( \alpha_6 \)-Expression is upregulated in lung myofibroblasts.** Next, we determined whether myofibroblast expression of \( \alpha_6 \) is altered in a human fibrotic disorder, IPF, and in a murine model of experimental lung fibrosis. Confocal immunofluorescent microscopy demonstrated high levels of \( \alpha_6 \)-expression in \( \alpha \)-SMA-positive lung myofibroblasts in fibroblastic foci of lung tissues of human subjects with IPF, as well as in fibrotic lesions following bleomycin lung injury in mice; in contrast, \( \alpha_6 \)-expression was primarily observed in the airway epithelium of normal human and mouse lungs (Fig. 3a). Primary lung myofibroblasts isolated from human subjects with IPF expressed significantly higher levels of \( \alpha_6 \) than primary lung fibroblasts isolated from control subjects (Fig. 3b,c). \( \alpha_6 \)-Integrin contains two structural variants, \( \alpha_6 \)A and \( \alpha_6 \)B, owing to alternatively spliced transcripts\(^{37}\). In addition, \( \alpha_6 \)-subunit pairs with either the \( \beta_1 \)- or \( \beta_3 \)-subunit to form functional integrin complexes. We demonstrated that although both \( \alpha_6 \)A and \( \alpha_6 \)B were expressed in human and mouse lung tissues at equivalent levels, lung (myo)fibroblasts primarily express the shorter \( \alpha_6 \)B isoform (Fig. 3d). Interestingly,
both normal lung fibroblasts and fibrotic lung myofibroblasts express similar levels of the β1-integrin subunit, while β4-protein expression in lung (myo)fibroblasts is not detectable (Fig. 3b,c). These results indicate that, in the context of fibrotic lung injury in vivo both in mice and humans, lung myofibroblasts express high levels of the α6-integrin; the α6β1 is the primary α6-integrin complex expressed by lung (myo)fibroblasts.

Inhibition of α6 protects against experimental lung fibrosis. To determine whether α6-expression in lung myofibroblasts plays a causal role in lung fibrogenesis, we generated conditional α6-knockout (α6-CKO) mice in which α6-gene is specifically deleted in collagen I-producing cells by intraperitoneal injection of tamoxifen. In pilot studies, we confirmed that tamoxifen treatment induces a time-dependent deletion of α6-expression in mouse lung fibroblasts (Fig. 4a). Almost complete deletion of α6-expression was observed after treatment of tamoxifen for 9 consecutive days. No significant reduction of α6-expression was observed in mouse whole-lung homogenates, suggesting that α6 deletion was mesenchymal cell-specific (Fig. 4a). Consistent with our previous findings (Fig. 3d), we observed that primary lung fibroblasts isolated from mice primarily express α6B isoform. On the basis of these time-course studies, we designed our experimental procedures as depicted in Fig. 4b: α6-CKO mice were given intratracheal bleomycin or saline on day 0. Since bleomycin-induced mouse lung fibrosis is characterized by acute lung injury and inflammation in the early phase (day 0–10) followed predominantly by lung fibrosis (day >14), we started intraperitoneal tamoxifen or corn oil (vehicle control for tamoxifen) treatment on day 5 post-bleomycin administration so that a complete knockout of α6 in lung fibroblasts would be expected to occur at ~14 days after lung injury; this minimizes potential effects of α6 deletion on the early phases of lung injury and inflammation. Mouse lungs were collected at day 21 and evaluated for lung fibrosis. Confocal immunofluorescent microscopy confirmed that α6A-positive lung myofibroblasts in corn oil-treated control mice expressed α6-integrin, whereas lung myofibroblasts in tamoxifen-treated mice did not (Fig. 4c). Mice with conditional deletion of the α6-gene during the post-inflammatory fibrotic phase of lung repair demonstrated marked attenuation of fibrotic responses, as assessed by trichrome staining of the lung for collagen (Fig. 4d), whole-lung hydroxyproline content (Fig. 4e), protein levels of fibronectin and αSMA in whole-lung homogenates (Fig. 4f), and micro-CT-based measurements of aerated lung volume, an inverse surrogate marker for pulmonary fibrosis (Fig. 4g). In addition, Mmp-2 expression was found in the area of αSMA-expressing lung myofibroblasts in both corn oil-treated control mice and tamoxifen-treated α6-CKO mice (Fig. 4h). Saline-treated WT and α6-CKO mice and bleomycin-treated α6-CKO mice showed intact continuous BMs, as demonstrated by immunostain of the BM component laminin. In contrast, the BM signals were largely disrupted in myofibroblast-enriched fibrotic regions of lungs from bleomycin-treated WT mice (Fig. 4i). Primary lung myofibroblasts isolated from bleomycin-treated α6-CKO mice (α6−/− MFBs) demonstrated reduced capacity for BM invasion as compared with primary lung myofibroblasts isolated from

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**Figure 3** | Lung myofibroblasts demonstrate increased α6-expression. (a) Frozen lung tissue sections obtained from failed normal human donors, patients with IPF, saline-treated mice and bleomycin-treated mice were double-stained for α6 (green) and αSMA (red). Nuclei were stained by DAPI (blue). Confocal immunofluorescent images were overlaid to show α6-expression in αSMA-positive lung myofibroblasts. Scale bar, 50 μm; scale bar, 20 μm for mouse with bleo images. (b) Comparison for α6-expression in lung (myo)fibroblasts isolated from patients with IPF (n = 6) and non-ILD control human subjects (n = 10) by immunoblot; Relative levels of α6-protein normalized to GAPDH expression. Results are the means ± s.d. Representative blots for α6-expression as well as β1- and β4-expression were shown. A549 cells were used as positive control for α6-expression in lung myofibroblasts by immunoprecipitation and immunoblot. (d) Identification of α6A and α6B expression in human and mouse lung tissues and fibroblasts by immunoblot; *P < 0.05, one-way analysis of variance.
bleomycin-treated WT mice (\(\alpha_6^+\)/MFBS); primary lung fibroblasts isolated from saline-treated \(\alpha_6\)-CKO mice (\(\alpha_6^-\)/FBs) and WT mice (\(\alpha_6^+\)/FBs) showed minimal invasion into the BM (Fig. 4j).

Since stiffness upregulates \(\alpha_6\)-expression through a c-Fos/c-Jun-dependent mechanotransduction pathway (Fig. 1), we determined whether pharmacological blockade of c-Fos/c-Jun pathway protects WT C57BL6 mice against bleomycin injury-induced experimental lung fibrosis. To minimize the potential effects of T-5224 on lung injury and inflammation, we started T-5224 or PVP (vehicle control) treatment at day 10 post-bleomycin administration (Fig. 5a). Mice treated with vehicle control showed \(\alpha_6\)-expression in \(\alpha\)SMA-expressing lung myofibroblasts, whereas \(\alpha_6\)-expression in lung myofibroblasts was greatly reduced in mice treated with T-5224 (Fig. 5b). In mice treated with bleomycin, phospho c-Jun was observed in the nuclei of \(\alpha\)SMA-positive lung myofibroblasts (Fig. 5c). In contrast, phospho c-Jun was absent in the lungs of saline-treated control mice. These data suggest that c-Fos/c-Jun signalling is activated in mouse lung fibrosis. Similar to genetic ablation of \(\alpha_6\) in lung mesenchymal cells, we observed that administration of T-5224 during the post-inflammatory fibrotic phase abrogated bleomycin injury-induced experimental lung fibrosis in mice (Fig. 5d, hydroxyproline content; Fig. 5e, immunoblot for fibronectin and \(\alpha\)SMA; Fig. 5f, Masson’s trichrome staining; Fig. 5g, micro-CT analysis of aerated lung volume). Control studies showed that tamoxifen had no effect on bleomycin-induced lung fibrosis in \(\text{Itga6}^-\) floxed mice (Supplementary Fig. 6a,b). Quantification of
inflammatory cells in bronchoalveolar lavage on day 14 demonstrated that post-inflammatory deletion of α6 in mesenchymal cells or T-5224 treatment did not alter the inflammatory response to bleomycin lung injury (Supplementary Fig. 6c,d).

Immunostaining of nuclear Ki-67, a cell proliferation marker, revealed that the vast majority of αSMA-positive lung myofibroblasts were non-proliferative (Supplementary Fig. 6e). Neither α6 deletion nor T-5224 treatment altered the proliferative rate of lung myofibroblasts. Altogether, these results provide strong support for a critical pro-fibrotic role for the mechanosensitive α6-integrin subunit, at least in part, by its capacity to mediate myofibroblast invasion.

**Discussion**

In this study, we identified that the α6-integrin subunit is a matrix stiffness-regulated mechanosensitive protein. Stiff matrix upregulates α6-integrin expression by ROCK-dependent activation of a c-Fos/c-Jun transcription complex in fibroblasts. Increased expression of α6-integrin is associated with enhanced capacity for lung myofibroblast invasion into the BM. We predict that α6-mediated myofibroblast-BM interactions bring

**Figure 6 | A model for mechanosensing α6 in the regulation of lung myofibroblast invasion into the BM.** Stiff/fibrotic matrix upregulates α6-expression by ROCK-dependent activation of c-Fos/c-Jun transcription complex. Interactions between α6-integrins, specifically α6β1-integrins, and the BM bring lung myofibroblasts into the close proximity to the BM. This facilitates MMP-2-mediated pericellular proteolysis of BM component collagen IV, leading to lung myofibroblast invasion.
myofibroblasts into the close proximity to the BM, which facilitates MMP-2-dependent pericellular proteolysis of collagen IV in the BM, thus promoting myofibroblast invasion (Fig. 6). Furthermore, we show that genetic deletion of α6 in (myo)fibroblasts or pharmacological blockade of the c-Fos/c-Jun mechanotransduction pathway, which regulates α6-expression, protects mice against experimental lung fibrosis. These in vivo studies suggest that targeting mechanosensing α6-integrins, specifically α6β1, may provide a novel anti-fibrotic strategy against pulmonary fibrosis. Previous studies have shown that mechanosensing by integrins may involve unmasking of cryptic sites within the cytoplasmic domains that allow for the binding of signalling molecules and/or transition of integrins from low- to high-affinity binding states.49. The present study, along with that of others40,41, suggests that regulation of integrin expression per se is an important mechanism for integrin-mediated mechanosensing.

We observe that α6-expression is increased in lung myofibroblasts of human IPF and bleomycin-induced lung fibrosis in mice. It has been reported that in IPF, lung epithelial cells express high levels of laminin adjacent to fibroblast foci.12 This finding is consistent with our observations that interactions between stiff matrix-regulated α6 in lung myofibroblasts and the BM mediate IPF myofibroblast invasion. Interestingly, BM-associated laminin-5 is associated with stromal fibroblastic reaction at the invasive front of lung adenocarcinoma, which may facilitate its invasiveness.43 In addition, human prostate cancer cells express high levels of α6-integrins; α6β1-integrins mediate prostate cancer metastasis to laminin-rich bone microenvironment.44. α6-Integrins also regulate the invasive phenotype of HT 1080 fibrosarcoma cells, and the levels of α6-integrins correlate with the degree of tumorigenicity of human neoplastic fibroblasts.12 In addition to the regulation of cell invasion, there is accumulating evidence that α6β1-integrins promote cell survival through both PI3K/Akt-dependent and -independent pathways.45-47. It has been reported that α6β1-integrins mediate collagen deposition in gingival fibroblasts, although the underlying mechanisms remain to be determined. Thus, it is possible that stiff matrix-induced α6-expression may not only regulate lung myofibroblast invasion, but contribute to their anti-apoptotic and matrix-remodelling properties as well.

We previously demonstrated that matrix stiffening activates RhoA/ROCK mechanosensitive signal pathway in lung (myo)fibroblasts.4 In this study, we showed that stiff matrix-induced phosphorylation of c-Jun and c-Fos requires ROCK activity. ROCK is a serine/threonine kinase.49 ROCK also activates serine/threonine kinases, p38 MAPK and PKC.50,51 It has been shown that both p38 MAPK and PKC induce phosphorylation of c-Fos and c-Jun in vitro and in vivo.52,53 It remains to be determined whether ROCK directly or indirectly mediates c-Fos and c-Jun phosphorylation in response to matrix stiffening. Although our studies implicate a definitive role for c-Fos/c-Jun in the ‘upstream’ regulation of α6-expression in response to matrix stiffness, the ‘downstream’ effects of α6 induction on cellular invasiveness may involve intracellular pathways that require further study. It has been shown that α6-integrins activate the small GTPase RAC by a PI3K-dependent mechanism.54 RAC activation promotes mesenchymal cell invasion into matrix barriers through mesenchymal–amoeboid transition.55,56 α6-Integrins also activate Src family kinase.57,58 Src family kinase signalling is known to promote cancer cell invasion.59

In this study, we found that lung (myo)fibroblasts primarily express α6B. Compared with α6A, α6B contains an alternative cytoplasmic domain that is 17 amino acids shorter and bears no sequence homology with α6A37. Whether the distinct cytoplasmic domain of α6B plays a functional role in the regulation of lung myofibroblast invasion into the BM, either by modulating myofibroblast adhesion to laminins in the BM and/or by activating cellular signals that mediate invasion is currently not known. Previous studies have shown that macrophages expressing α6Aβ1 or α6Bβ1 differ markedly in their morphology and migration on laminin matrix.60 Macrophage adhesion to laminin matrix is regulated by phosphorylation of the cytoplasmic domain of α6-integrins at the serine residues.61 It has also been reported that α6Aβ1 and α6Bβ1 differentially regulate tyrosine phosphorylation of paxillin on laminin matrix.62

AP-1 is a heterodimer composed of proteins belonging to the c-Fos, c-Jun, ATF and JDP families. We demonstrated that the prototypic members of AP-1 transcription factor family, c-Fos and c-Jun, mediate stiff matrix-induced α6-gene expression. In previous studies, Eferl et al.59 have shown that Fos-related Fra2 transgenic mice develop spontaneous fibrosis in various organs with predominant involvement of the lung. Fichtner-Feigl et al.32 have reported that the Fra2/c-Jun complex mediates IL-13/IL-13α-gene expression. In vivo ROCK inhibition of the TGF-β1 promoter in bleomycin-induced mouse lung fibrosis. However, in our studies, Fra2 does not appear to be involved in matrix stiffness-regulated α6 expression. Thus, distinct AP-1 transcription factor complexes may be responsible for different components of fibrogenic signalling pathways. Since AP-1 is a heterodimer, blocking c-Fos/c-Jun with T-5224, a selective Fos inhibitor, may interrupt the function of other Fos-containing AP-1 complexes. Therefore, T-5224 treatment might not only block stiff matrix-induced α6-expression and myofibroblast invasion, but other potential fibrogenic signals regulated by Fos-containing AP-1 complexes.

MMPs, including MMP-2 and MMP-9 of type IV collagenases, are critical players in the pathogenesis of human IPF.61 In this study, we demonstrated that MMP-2 is the primary type IV collagenase that mediates matrix stiffness-regulated IPF lung myofibroblast invasion into the BM. Interestingly, matrix stiffness regulates MMP-9 expression at the mRNA level, although MMP-9 activity is not detected. In addition to MMP-9, matrix stiffness also regulates mRNA expression of MMP-11, MMP-12 and MMP-16 (Table 1). AP-1 has been shown to mediate MMP expression in response to phorbol myristate acetate and cytokines.62. Although bioinformatics analyses identified potential AP-1-binding sites in the promoter region of MMP-9, MMP-11, MMP-12 and MMP-16, we found that neither T-5224 nor AP-1 decoy oligodeoxynucleotides blocked matrix stiffness-regulated MMP-9, MMP-11, MMP-12 and MMP-16 mRNA expression (Supplementary Fig. 5e). These data suggest that matrix stiffness-regulated gene expression of MMP-9, MMP-11, MMP-12 and MMP-16, unlike the integrin α6 subunit, may occur via AP-1-independent mechanisms.

It is currently unclear if matrix stiffness is a cause or consequence of organ fibrosis.63 There is accumulating evidence that mechanical interactions between myofibroblasts and stiffened matrix provide a feed-forward mechanism that maintains pro-fibrotic myofibroblast phenotypes and, therefore, perpetuation of fibrosis.1,3–6. In rat carbon tetrachloride model of liver fibrosis, it has been observed that matrix stiffness increases before myofibroblast differentiation and fibrosis.64,65 This early increase in liver stiffness can be blunted by inhibition of collagen cross-linking enzymes of the lysyl oxidase family.64 These interesting findings suggest that changes in the mechanical properties of the ECM may not only sustain myofibroblast phenotype but contribute to the emergence of myofibroblasts in early liver fibrosis. Altogether, these studies imply that new therapies that target deleterious mechanical signals may be effective in preventing or arresting the progression of fibrosis.
In summary, the findings from this study support an essential role of the mechanosensing α6-integrin in mediating myofibroblast invasion and lung fibrosis following injury. Importantly, this novel mechanosensing pathway represents a target for developing new anti-fibrotic therapeutic strategies. Strategies for blocking the deleterious function of mechanosensing α6 might include the development of specific antibodies against fibroblast α6-integrins, specifically α6β1, or pharmacological disruption of the mechanotransduction pathway involved in α6-expression. Interestingly, miR-29, an anti-fibrotic master regulator capable of blocking and reversing pulmonary fibrosis, directly targets both α6-integrin and laminin25. Future studies that focus on targeting the invasive phenotype of myofibroblasts, in addition to other pro-fibrotic properties such as apoptosis-resistance, may prove to be effective in treating fibrotic disorders.

**Methods**

**Lung fibroblast isolation and treatments.** Human lung fibroblasts were established from tissue samples from patients undergoing lung transplantation. Previous studies have shown that lung myofibroblasts isolated from patients with IPF acquire an invasive phenotype, whereas normal human lung fibroblasts do not invade26. IPF lung myofibroblasts were used in this study. The studies involving human subjects were approved by institutional review board at the University of Alabama at Birmingham. Participants have been provided with written informed consent. Lung fibroblast isolation, culture, transfection, sorting and treatment were described in Supplementary Methods.

**Matrigel invasion assay.** Fibrotic lung fibroblasts were cultured on soft (1 kPa) and stiff (20 kPa) PA gels for 48 h. Cells were detached from PA gels by trypsinization. An equal number of living cells (1 × 10^5 cells per chamber) derived from soft and stiff matrix were plated in Matrigel invasion chambers (BD Biosciences, San Jose, CA, USA). Cell invasion was measured at 7 h after incubation on Matrigel to minimize the potential effect of differential matrix stiffness on fibroblast proliferation1. Non-invading cells at the bottom of invasion chambers were swiped with cotton swabs. Invading cells on the other side of Matrigel membrane were stained with 0.5% crystal violet for 30 min. The number of invading cells was counted under a Nikon Eclipse TE 300 microscope equipped with Spot Insight CCD camera. Invasion index was calculated as the ratio of the percent invasion of test cells (cells cultured on soft and stiff PA gels) over the percent invasion of control cells (cells cultured on regular tissue culture plates). In a second approach, an equal number of fibrotic lung fibroblasts were seeded on soft or stiff PA gels. Cells were allowed attachment for 1 h. Cells together with PA gels were transferred to Matrigel invasion chambers with the apical side of cells in close contact with Matrigel (Supplementary Fig. 3). Invading cells were counted at 48 h.

**Proteolytic degradation of collagen IV in the BM.** Six-well Matrigel invasion chambers were incubated with DQ-collagen IV (Molecular Probes, Eugene, OR) diluted in serum-free DMEM at a final concentration of 25 μg mL−1 at 37 °C in dark overnight. The chambers were briefly rinsed with serum-free DMEM. Fibrotic lung fibroblasts were trypsinized from stiff matrix. In all, 1 × 10^5 cells were seeded into each invasion chamber in the presence or absence of NKI-GoH3 (10 μg mL−1), α6 siRNA and MMP-2/2-MMP-9 Inhibitor I (25 μM). Cells were then incubated in a CO2 incubator at 37 °C for 3 h. Proteolytic degradation of DQ-collagen IV in the BM was imaged with confocal laser-scanning microscopy as described below.

**Crispr.** Two 20-base sgRNAs were designed to target AP-1-binding TREs at −2,873/−2,879 nt and −4,484/−4,854 nt in human α6-promoter, respectively. AP1sgRNA1 (5’-CTAAGAATCCTTAGGCCTTGGAAC-3’) binds to the plus-strand sequence at −4,481/−4,486 nt near the distal TRE1 in human α6-promoter. AP1sgRNA2 (5’-CACCACACTCTGTATCAAA-3’) binds to the minus-strand DNA at −2,924/−2,943 nt near the proximal TRE2 (Fig. 1h). Both of the sequences were cloned into pX333-Addgene (Addgene) to obtain pX333-AP1sgRNA1-AP1sgRNA2 plasmid. A DNA fragment encoding dCas9-BFP-KRAB domain was amplified by PCR from pHIR-SFFV-dCas9-BFP-KRAB (Addgene). The fragment was subcloned into pX333-AP1sgRNA1-AP1sgRNA2-ΔdCas9-BFP-KRAB plasmid. The latter plasmid and pX333 empty vector were transfected into IPF lung myofibroblasts using a Nucleofector device (Amaxa) as previously described4.

**Immunofluorescence and confocal laser-scanning microscopy.** Eight micro-metre cryostat sections were rehydrated in phosphate-buffered saline for 10 min. Tissue sections were blocked with 5% normal goat serum and co-stained with anti-α5 (Sigma, St Louis, MO, Cat#A2547, 1:200 dilutions) and anti-α6 (Abcam, Cambridge, MA, Cat#14-0495, 1:300 dilutions) antibodies diluted in phosphate-buffered saline containing 1% goat serum, 0.3% Triton X-100 and 0.1% sodium azide according to manufacturer’s instructions. Fluorochrome-conjugated secondary antibodies (SouthernBiotech, Birmingham, AL) were used according to the manufacturer’s recommendation. Nuclei were stained with DAPI (Thermo Fisher Scientific, Waltham, MA). Fluorescent signals were detected using a confocal laser-scanning microscope Zeiss LSM710 confocal microscope equipped with a digital colour camera (Oberkochen, Germany). All fluorescent images were generated using sequential laser scanning with only the corresponding single-wavelength laser line, activated using acousto-optical tunable filters to avoid cross-detection of either one of the fluorescence channels.

**Animals and experimental protocol.** The animal studies were performed in accordance with the NlH guidelines for Care and Use of Laboratory Animals. Animal usage and bleomycin protocols were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. To generate mesenchymal cell-specific Itgα6/−/− mice, C57BL/6-Itgα6/−/− mice (a gift from Dr Elisabeth Georges-Labouesse, Institut de Genetique et de Biologie Moleculaire et Cellulaire, Illkirch, France) were crossed with B6C7Bl6 mice carrying a tamoxifen-inducible Cre-recombinase (Cre-ERT2) under the control of a regulatory sequence from procollagen I gene (The Jackson Laboratory, Bar Harbor, ME). Six- to eight-week-old female conditional Itgα6/−/− mice and WT C57Bl6 mice were used in this study. Bleomycin sulphate (Almirall, Barcelona, Spain) was dissolved in sterile saline solution and intratracheally instilled into mice by a Stepper Repipette Pipettor (Tridak, Torrington, CT) as a single dose in 50 μl saline solution per animal (1 U kg−1 bodyweight). Control mice received 50 μl saline. For tamoxifen (Sigma, St Louis, MO) treatment, a dosage of 50 mg kg−1 bodyweight per day over 9 days or an equal volume of corn oil (vehicle for tamoxifen) was injected intraperitoneally into conditional Itgα6/−/− mice, 5 days after bleomycin administration. For T-5224 (Apexbio, Houston, TX) treatment, a dosage of 30 mg kg−1 bodyweight per day or an equal volume of PVP (vehicle for T-5224) was given to WT C57Bl6 mice daily by oral gavage. 10 days after bleomycin administration. Mice were killed at 21 days. Lung tissues were collected and used for histochemical and immunofluorescent analyses, micro-CT scans and isolation of lung fibroblasts.

**Statistical analysis.** Statistical differences among treatment conditions were determined using one-way analysis of variance (Newman–Keuls method for multiple comparisons). Values of P < 0.05 or P < 0.01 were considered significant.

**Data availability.** All relevant data will be made available on request and/or are included with the manuscript (as figure source data or Supplementary Information files). Additional information is detailed in the Supplementary Methods.

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Author contributions
Y.Z., H.C. and J.Q. designed the study; H.C., J.Q., X.H., A.K., L.Z., N.Y. and A.V. performed the experiments; H.C., J.Q. and Y.Z. analysed the data; V.J.T., V.B.A., G.L. and Q.D. provided experimental materials and participated in discussion; H.C. and Y.Z. wrote the manuscript; V.J.T. and Y.Z. revised the manuscript.

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