Overexpression of c-Met and CD44v6 Receptors Contributes to Autocrine TGF-β1 Signaling in Interstitial Lung Disease*

Shibnath Ghatak1, Galina S. Bogatkevich5, Ilia At内shvili5, Tanjina Akter5, Carol Feghali-Bostwick2, Stanley Hoffman1, Victor M. Fresco1, John C. FuchsE, Richard P. Visconti1, Roger R. Markwald1, Subhas B. Padhye4, Richard M. Silver1, Vincent C. Hascall1, and Suniti Misra1,2

From the 4Department of Regenerative Medicine and Cell Biology and the 4Division of Rheumatology and Immunology, Department of Medicine, Medical University of South Carolina, Charleston, South Carolina 29425, the 4Interdisciplinary Science and Technology Research Academy (ISTRA), Abeda Inamdar College, University of Pune, Azam Campus, Hidayatullah Road, Camp Pune 411001, India, and the 1Department of Biomedical Engineering/ND20, Cleveland Clinic, Cleveland, Ohio 44195

Background: CD44v6 and c-Met contribute to TGF-β1 signaling in interstitial lung disease (ILD). CD44 contains variable exon 6 (CD44v6) and its ligand hyaluronan (HA) alter cellular function in response to interaction between CD44v6 and HA. TGF-β1 is the crucial cytokine that induces fibrotic action in ILD fibroblasts (ILDFb). We have identified an autocrine TGF-β1 signaling that up-regulates both Met and CD44v6 mRNA and protein expression. Western blot analysis, flow cytometry, and immunostaining revealed that CD44v6 and Met colocalize in fibroblasts and in tissue sections from ILD patients and in lungs of bleomycin-treated mice. Interestingly, cell proliferation induced by TGF-β1 is mediated through Met and CD44v6. Further, cell proliferation mediated by TGF-β1/CD44v6 is ERK-dependent. In contrast, action of Met on ILDFb proliferation does not require ERK but does require p38MAPK. ILDFbs were sorted into CD44v6+/Met+ and CD44v6+/Met− subpopulations. HGF inhibited TGF-β1-stimulated collagen-1 and α-smooth muscle cell actin expression in both of these subpopulations by interfering with TGF-β1 signaling. HGF alone markedly stimulated CD44v6 expression, which in turn regulated collagen-1 synthesis. Our data with primary lung fibroblast cultures with respect to collagen-1, CD44v6, and Met expressions were supported by immunostaining of lung sections from bleomycin-treated mice and from ILD patients. These results define the relationships between CD44v6, Met, and autocrine TGF-β1 signaling and the potential modulating influence of HGF on TGF-β1-induced CD44v6-dependent fibroblast function in ILD fibrosis.

The hepatocyte growth factor (HGF) and the HGF receptor Met pathway are important in the pathogenesis of interstitial lung disease (ILD). Alternatively spliced isoforms of CD44 containing variable exon 6 (CD44v6) and its ligand hyaluronan (HA) alter cellular function in response to interaction between CD44v6 and HGF. TGF-β1 is the crucial cytokine that induces fibrotic action in ILD fibroblasts (ILDFb). We have identified an autocrine TGF-β1 signaling that up-regulates both Met and CD44v6 mRNA and protein expression. Western blot analysis, flow cytometry, and immunostaining revealed that CD44v6 and Met colocalize in fibroblasts and in tissue sections from ILD patients and in lungs of bleomycin-treated mice. Interestingly, cell proliferation induced by TGF-β1 is mediated through Met and CD44v6. Further, cell proliferation mediated by TGF-β1/CD44v6 is ERK-dependent. In contrast, action of Met on ILDFb proliferation does not require ERK but does require p38MAPK. ILDFbs were sorted into CD44v6+/Met+ and CD44v6+/Met− subpopulations. HGF inhibited TGF-β1-stimulated collagen-1 and α-smooth muscle cell actin expression in both of these subpopulations by interfering with TGF-β1 signaling. HGF alone markedly stimulated CD44v6 expression, which in turn regulated collagen-1 synthesis. Our data with primary lung fibroblast cultures with respect to collagen-1, CD44v6, and Met expressions were supported by immunostaining of lung sections from bleomycin-treated mice and from ILD patients. These results define the relationships between CD44v6, Met, and autocrine TGF-β1 signaling and the potential modulating influence of HGF on TGF-β1-induced CD44v6-dependent fibroblast function in ILD fibrosis.

Interstitial lung disease (ILD) is a large group of fibrotic lung diseases, including systemic sclerosis (SSc)-associated lung disease and idiopathic pulmonary fibrosis. ILD is characterized by recurrent injury to the alveolar epithelium, resulting in distortion of its architecture and a failure of tissue repair. Tissue fibrosis is regarded as a final consequence when organs face continuous injury. In response to stimuli from injured areas, affected tissues initially undergo a series of steps typical of wound healing. Injury is followed by 1) damage to the basement membrane of the epithelial/endothelial barrier; 2) release of TGF-β1, the major fibrogenic cytokine; 3) overexpression of hyaluronan (HA); 4) recruitment of inflammatory cells; 5) induction of reactive oxygen species; 6) activation of collagen-producing cells; and 7) matrix activation of myofibroblasts. When the cause of continuous injury is removed, the basement membrane of the epithelial/endothelial barrier can regain its integrity, and reversal of fibrosis can occur. However, when exposed to chronic injuries, the wound-healing process fails, leading to tissue fibrosis, which is characterized by the overproduction of extracellular matrix, which ultimately causes fibrotic lesions and tissue scarring.

*This work was supported by 1R03CA167722-01A1 (to S. M. and S. G.); P20RR021949 (to S. G.), P20RR16434 (to S. M., S. G., and R. R. M.), P20RR05461-05 (to S. G. and R. R. M.), R01-HL03375-24 (to S. M., S. G., and R. R. M.), P01HL107147 and 1P30AR050953 (to V. C. H.), EPS 0903795 (to S. G.), P20RR016434 (to S. M., S. G., and R. R. M.), P20RR021949 (to S. M., S. G., and R. R. M.), and Multidisciplinary Clinical Research Center 39919 (to S. G. and S. M.).

1To whom correspondence may be addressed: Dept. of Regenerative Medicine and Cell Biology, Medical University of South Carolina, 173 Ashley Ave., Charleston, SC 29425. Tel.: 843-792-8642; Fax: 843-792-2965; E-mail: ghatak@musc.edu.

2To whom correspondence may be addressed: Dept. of Regenerative Medicine and Cell Biology, Medical University of South Carolina, 173 Ashley Ave., Charleston, SC 29425. Tel: 843-792-8642; Tel: 843-792-2965; Fax: 843-792-0664; E-mail: misra@musc.edu.

3The abbreviations used are: ILD, interstitial lung disease; HGF, hepatocyte growth factor; HA, hyaluronan; CD44v6, CD44 containing variable exon 6; ILDFb, ILD fibroblast; NLFb, normal lung fibroblast; α-SMA, α-smooth muscle cell actin; SSc, systemic sclerosis; collagen-1, collagen type I; Met, c-Met; control shRNA, pSicoR-scrambled shRNA; CD44v6 shRNA, pSicoR-CD44v6 shRNA; Met shRNA, pSicoR-Met shRNA; qRT-PCR, quantitative RT-PCR; pERK, pAKT, and pp38, phosphorylated ERK, AKT, and p38MAPK, respectively.

The hepatocyte growth factor (HGF) and the HGF receptor Met pathway are important in the pathogenesis of interstitial lung disease (ILD). Alternatively spliced isoforms of CD44 containing variable exon 6 (CD44v6) and its ligand hyaluronan (HA) alter cellular function in response to interaction between CD44v6 and HGF. TGF-β1 is the crucial cytokine that induces fibrotic action in ILD fibroblasts (ILDFb). We have identified an autocrine TGF-β1 signaling that up-regulates both Met and CD44v6 mRNA and protein expression. Western blot analysis, flow cytometry, and immunostaining revealed that CD44v6 and Met colocalize in fibroblasts and in tissue sections from ILD patients and in lungs of bleomycin-treated mice. Interestingly, cell proliferation induced by TGF-β1 is mediated through Met and CD44v6. Further, cell proliferation mediated by TGF-β1/CD44v6 is ERK-dependent. In contrast, action of Met on ILDFb proliferation does not require ERK but does require p38MAPK. ILDFbs were sorted into CD44v6+/Met+ and CD44v6+/Met− subpopulations. HGF inhibited TGF-β1-stimulated collagen-1 and α-smooth muscle cell actin expression in both of these subpopulations by interfering with TGF-β1 signaling. HGF alone markedly stimulated CD44v6 expression, which in turn regulated collagen-1 synthesis. Our data with primary lung fibroblast cultures with respect to collagen-1, CD44v6, and Met expressions were supported by immunostaining of lung sections from bleomycin-treated mice and from ILD patients. These results define the relationships between CD44v6, Met, and autocrine TGF-β1 signaling and the potential modulating influence of HGF on TGF-β1-induced CD44v6-dependent fibroblast function in ILD fibrosis.
Although numerous factors as described above have a significant role in fibrogenesis, release of TGF-β1 is a key event in pathogenesis of fibrosis. In vitro, TGF-β1 has been shown to stimulate myofibroblast cell activation, which leads to overproduction of matrix components in fibrotic diseases (1, 2). In accordance with this, blockade of TGF-β1 signaling with neutralizing antibody has been shown to be effective in counteracting fibrosis in animal models (3, 4). In addition, blockade of TGF-β1 signaling abolished the overexpression of collagen mRNA in cultured ILD fibroblasts (ILDfbs) (5–7), indicating that the TGF-β1/Smad-dependent pathway is crucial for the intrinsic up-regulation of collagen genes that is observed in ILDfbs.

In the presence of fibrogenic stimuli in culture, such as TGF-β1, or of certain types of extracellular matrices (fibronectin and collagen type I (collagen-1)), mesenchymal lineage-derived fibroblasts rapidly acquire a myofibroblastic phenotype, including expression of α-SMA and secretion of collagen-1 (8, 9). Extensive studies indicate that activated myofibroblasts are the major effector cells responsible for an inexorable expression of interstitial matrix components, such as collagen, which can directly result in hyperproliferation of fibroblasts (9–11).

HA levels are elevated in serum of ILD patients (12, 13) and in the lung after bleomycin treatment (14–16). CD44 participates in the activation of leukocytes and parenchymal cells in areas of inflammation, suggesting a role for CD44 in tissue remodeling and fibrosis (17, 18). Fibroblast recruitment is critical to wound healing, and CD44 is important in both maintaining the integrity of the actin cytoskeleton (19–21) and in facilitating an organized, directional migratory response to injury (22). Hence, myofibroblast activation is often regarded as a central event that has a critical role in the onset and progression of fibrosis.

HA is a ubiquitous connective tissue glycosaminoglycan, which is present in vivo as a high molecular weight component of extracellular matrices. Increased deposition of interstitial HA has also been correlated with tissue function in progressive fibrosis, including ILD (12, 13, 23). Progressive ILD requires the generation of an invasive myofibroblast phenotype that requires TGF-β1, where hyaluronan synthase 2 and CD44 are critical downstream components of TGF-β1-induced fibrosis (14). Interaction of HA with alternatively spliced isoforms of CD44 that contain variable exon 6 (CD44v6) alter cellular function in response to various growth factors and cytokines (24). A recent study suggests that CD44v6 can sustain its own synthesis through a positive feedback loop that couples CD44v6 and MAPK through the HGF receptor Met, whose phosphorylation activates MAPK. Previous studies indicated that in SSc dermal fibroblasts and in ILDfbs, HGF exerts antifibrotic effects through the HGF/Met pathway by increasing MMP1 production (1, 25–27). In addition, HGF is also capable of preventing α-SMA expression in kidney fibrosis both in vitro and in vivo (28–30). The functional significance of alterations in the expression of Met associated with ILD injury, however, is not clear. Importantly, involvement of the closely connected CD44v6 with TGF-β1-induced Met has not been studied in ILD. The aim of the current study was to define the relationships between CD44v6, Met, and TGF-β1 autoregulation and the potential modulating influence of HGF on TGF-β1-induced CD44v6-dependent signaling and function in ILD fibrosis.

EXPERIMENTAL PROCEDURES

Materials

Dulbecco’s modified Eagle’s medium (DMEM) low glucose, glutamine, and pyruvate were from Life Technologies. Fetal bovine serum was from Atlanta Biologicals, and l-glutamine, gentamicin sulfate, and amphotericin B were from Hyclone. Actinomycin D, cycloheximide, Nonidet P-40, EGTA, sodium orthovanadate, glyceral, phenylmethylsulfonyl fluoride, leupeptin, pepstatin A, aprotinin, and HEPES were purchased from Sigma. Recombinant human TGF-β1 was purchased from R&D Systems (Minneapolis, MN). The antibodies against c-Met, CD44, collagen-1, HSP47, Smad7, phosphorylated Smad2, TGF-βRI, α-SMA, pERK, ERK, GAPDH, β-actin, horseradish peroxidase-linked anti-rabbit and anti-mouse antibodies, and Luminol reagent were purchased from commercial sources (Santa Cruz Biotechnology, Inc., Abcam, Ebioscience, Thermo Fisher, Cell Signaling Technology, and Southern Biotechnology Associates Inc.). The bromodeoxyuridine kit was purchased from Millipore Corp. CD44v6 siRNA, Met siRNA, and TGF-β1 siRNA oligonucleotides were synthesized by ID Technology.

Management of Animals and Human Lung Samples

ILDfbs, isolated from lung tissues, were obtained from Dr. Galina Bogatkevich, Dr. R. M. Silver, and Dr. Carol Feghali-Bostwick. The lung tissues were obtained from autopsy and from explants following lung transplantation from three scleroderma patients. Normal lung tissues were isolated from three age- and gender-matched normal healthy subjects whose lungs were not used for transplantation.

6-Week-old mice (C57BL/6 strain) were obtained from Jackson Laboratories. Bleomycin (0.05 units/20 g of animal) was instilled intratracheally. All animal care and experimentation were done in accordance with the institutional animal care and use committee protocol (AR 3220) approved by the Medical University of South Carolina according to the rules of the National Institutes of Health. Lung tissues at 21 days after vehicle or bleomycin (Sigma) instillations were perfused with Z-Fix (Anatech Ltd.) and processed for paraffin sections.

Cell Culture

Fibroblasts were isolated and cultured as reported previously (31). Briefly, lung tissues were diced (≈0.5 × 0.5-mm pieces) and cultured in DMEM with normal glucose, glutamine, and pyruvate (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, gentamicin sulfate (50 μg/ml), and amphotericin B (5 μg/ml) at 37°C in 10% CO2. The medium was changed every 3 days to remove dead and non-attached cells until fibroblasts reached confluence. Monolayer cultures were maintained in the same medium. Lung fibroblasts were used between the second and fourth passages in all experiments. The purity of isolated lung fibroblasts was determined by crystal violet staining and by immunofluorescence staining using monoclonal antibody 3C4 against human fibroblasts as
described previously (26). All of the treatments and transfection experiments were done with cells that were serum-starved for 24 h.

**Cell Lysis and Immunoblotting**

Fibroblasts were cultured until they were confluent. Cells were washed twice at 4 °C with 1× phosphate-buffered saline (PBS), harvested with 0.05% Versene, and then washed in cold PBS again. The cells were pelleted by centrifugation at 5000 × g for 2 min at 4 °C. The pellets were treated with the lysis buffer containing 1% Nonidet P-40, 0.5 mM EGTA, 5 mM sodium orthovanadate, 10% (v/v) glycerol, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, and 50 mM HEPES, pH 7.5. The lysates were clarified by centrifugation at 12,000 × g for 10 min at 4 °C and then stored at −80 °C as described previously (32–39). Cell lysates (normalized for protein concentration) were analyzed by immunoblotting as described previously (34–36, 40). The proteins on the blots were analyzed with antibodies for c-Met, CD44v6, Met, and TGF-β1 from Santa Cruz (Santa Cruz Biotechnology, Inc.) following treatment with horseradish peroxidase standards and detected by Luminol reagent (Santa Cruz Biotechnology, Inc.).

**Nuclear Protein Extract Preparation and Western Blotting for Phosphorylated Smad2**

Nuclear extracts were prepared from ~1–1.5 × 10^7 fibroblasts. Cells were washed in ice-cold 1× PBS and incubated for 10 min on ice in 200 μl of buffer containing 10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride. At the end of the incubation, 0.1 volumes of 1% Nonidet P-40 was added, and the lysates were immediately centrifuged at low speed (~350 × g) at 4 °C for 2 min. The supernatants were discarded, and the pelleted nuclei were resuspended in 50 μl of a lysis buffer containing 20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol. After incubation for 15 min at 4 °C with vigorous shaking, the lysed nuclei were centrifuged at 16,000 × g for 10 min to clear the debris. The supernatants were aliquoted, snap-frozen, and stored at −80 °C until further use. 30-μg protein samples were separated using 4–15% gradient polyacrylamide gels, transferred to nitrocellulose membranes, and probed with rabbit anti-phospho-Smad2 (pSmad2) (Ser-465/467; 1:1000) and reprobed for β-actin (1:1500) (antibodies from Cell Signaling Technology).

**RNA Silencing**

Control siRNA (scrambled siRNA) and CD44v6 siRNA were prepared as described previously (40, 41). Met siRNA and TGF-β1 siRNA were from ID Technology.

**CD44v6 shRNA and Met shRNA Cloning in pSicoR Vectors**

Double-stranded oligonucleotide cassettes for control shRNA (scrambled shRNA), CD44v6 shRNA, and Met shRNA were prepared. The linearized pSicoR vectors were ligated to the double-stranded oligonucleotide cassettes (40). The resulting pSicoR-CD44v6 shRNA (CD44v6 shRNA) and pSicoR-Met shRNA (Met shRNA) transfectants constitutively silence respective CD44v6 and Met genes in the cells. pSicoR-scrambled shRNA (control shRNA) transfectants were used as a control to the above shRNA transfectants.

**Cell Proliferation Assays**

**Immunofluorescence Using BrdU Incorporation into Normal Lung Fibroblasts (NLFbs) and ILDFbs**—Cell proliferation was determined in immunofluorescence-based assays by BrdU incorporation in NLFb and ILDFb cultures treated with or without TGF-β1 using Millipore’s HCS213 assay kit according to the manufacturer’s instructions. Briefly, cells (4 × 10^5) were plated in each well of a 96-well plate. 48 h after incubation at 37 °C in 5% CO₂, the cells were serum-starved in serum-free medium (QBSF-51). TGF-β1 at the concentrations indicated under “Results” was added to the wells, followed by incubation for 24 h. 20 μM BrdU was then added to the wells, followed by incubation for 4 h. The cells were washed, fixed, and then incubated with primary mouse anti-BrdU antibody for 1 h at room temperature. The cells were washed in immunofluorescence buffer and then incubated with a secondary antibody linked to Alexa Fluor® 555 and Hoechst 33362 in nuclear stain solution for 1 h at room temperature protected from light. After washing successively with immunofluorescence buffer and wash buffer, images were taken using a Leica microscope.

**Cellular ELISA Using BrdU Incorporation**—Cell proliferation was measured by the BrdU cellular ELISA kit based on the incorporation of the pyrimidine analog BrdU into the DNA of proliferating cells that are cultured in microtiter plates. After its incorporation into DNA, BrdU in the cells was detected by anti-BrdU monoclonal antibody and added to a 96-well plate. Fibroblasts (20,000 cells) were cultured in a 96-well plate at 37 °C. BrdU at 20 μM was added to wells during the final 2–18 h of culture. Cells were then fixed and permeabilized, and the DNA was denatured to enable antibody binding to the incorporated BrdU. Detector mouse anti-BrdU monoclonal antibody was pipetted into the wells and allowed to incubate for 1 h. Unbound antibody was then washed away, and horseradish peroxidase-conjugated goat anti-mouse IgG was added, which binds to the detector antibody. The horseradish peroxidase catalyzes the conversion of the chromogenic substrate tetramethylbenzidine to form a blue solution (or yellow after the addition of stopping reagent). The absorbance at 450 nm was quantified in a Bio-Tek microplate reader and reflects the relative amount of incorporated BrdU in the cells.

**Quantitative Real-time PCR (qRT-PCR) of CD44s, CD44v6, and Met from NLFbs and ILDFbs**

Total RNA was isolated from NLFbs and ILDFbs and from FACS-sorted CD44v6+/−/Met+ subpopulations from ILDFbs after various treatments and transfections as mentioned in the figure legends for each specified experiment using the RNeasy minikit (Qiagen) according to the standard protocol provided by the manufacturer, with on-column DNA digestion. RNA integrity and concentration were analyzed using Bioanalyzer,
and 1 µg of RNA was retrotranscribed into cDNA using the First Strand cDNA synthesis kit from Roche Applied Science. SYBR Green technology (Bio-Rad) was used for all qRT-PCR experiments. Amplification was done with a qRT-PCR analyzer (Bio-Rad Biosystems). The PCR mixture (25 µl) contained 12.5 µl of 2X SYBR Green PCR Master Mix (Bio-Rad), 5 µl of diluted RT product (1:20), and 0.5 µm sense and antisense primer sets. The primers used were as follows: 5'-AGAAGGT-GTGGGCAAGAAA-3' (forward) and 5'-AAATGCACCACT-TTCTTGAGA-3' (reverse) for CD44s, 5'-AGAACAGTGG-TTGGGACAC-3' (forward) and 5'-GAATGGGATTCCTTCTG-G-3' (reverse) for CD44v6, 5'-CAGGCCGTTCCAAGT-GAG-3' (forward) and 5'-ATCATACTGAGCAGCAAGTTCCC-3' (reverse) for COL1A1 (collagen type-1α1); 5'-AGCAACATGG- CCAATTTTACAAGAG-3' (forward) and 5'-ATCATACTGAGCAGCAAGTTCCC-3' (reverse) for COL1A2 (collagen type-1α2); 5'-AAAGGGCATTTTGGTTGTG-3' (forward) and 5'-GATGATTTCCCTCGGTACAA-3' (reverse) for c-Met.

The qRT-PCR assays were done in three individual experiments with triplicate samples using standard conditions. After sequential incubations at 50 °C for 2 min and 95 °C for 10 min, respectively, the amplification protocol consisted of 50 cycles of denaturing at 95 °C for 15 s, annealing, and extension at 60 °C for 60 s. The standard curve was made from a series dilution of template cDNA. Expression levels of c-Met and CD44v6 mRNA were calculated after normalization with the housekeeping gene β-actin.

Transgenic Transfection Using NLFbs and ILDFbs

All transfections were done using Lipofectamine (Invitrogen) in cultures at ~75% confluence. After transfection, the cultures were grown for another 72–96 h for analyses.

Determination of HGF in the Culture Medium

Fibroblasts (1 × 10^5 cells/well) were cultured in 6-well culture plates with DMEM plus 10% FBS. At confluence, culture medium was discarded, and each well was washed with PBS twice. Serum-free medium (QBSF-51; Sigma) was then added, followed by incubation for various times. The supernatants were collected and stored at ~80 °C until use. HGF concentrations in culture supernatants were measured by an ELISA from R&D Systems.

Determination of TGF-β1 in the Culture Medium

Fibroblasts (1 × 10^5 cells/well) were cultured in 6-well culture plates with DMEM plus 10% FBS. At confluence, culture medium was discarded, and each well was washed with PBS twice. Serum-free medium (QBSF-51; Sigma) was then added and incubated for various times. The supernatants were collected and stored at ~80 °C until use. We measured the bioactive form of the TGF-β1. The TGF-β1 present in the culture supernatant was first converted into the bioactive form by acid treatment and neutralization and then estimated by a sandwich ELISA kit (Enzo Life Sciences) according to the manufacturer’s instructions. Briefly, culture supernatant containing TGF-β1 (100 µl) was first activated by acid treatment for 10 min with 20 µl of 1N HCl and neutralized by 20 µl of 1.2N NaOH. 0.5 M HEPES. To eliminate matrix interference in the assay, 140 µl was diluted with an equal volume of assay buffer. A sample of serum-supplemented cell culture medium treated in parallel was analyzed and used as control. Standard TGF-β1 was serially diluted. Samples of controls and standards were added to wells coated with TGF-β1 monoclonal antibody to capture the TGF-β1. Bound TGF-β1 was then sandwiched by a polyclonal antibody against TGF-β1. The sandwich complex was treated with horseradish peroxidase-conjugated secondary antibody against the polyclonal antibody. The enzyme was allowed to react with 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide. The reaction was stopped by adding alkali, and the color was read at 450 nm. The TGF-β1 content in the supernatant was calculated by subtracting the control reading and comparing with a standard curve.

Immunohistochemical Staining in Lung Sections

Lung sections from bleomycin- and saline-treated mice and from ILD patients and normal subjects were deparaffinized using standard procedures and permeabilized with 0.1% Triton X-100 in PBS. Met, CD44v6, and collagen-1 were localized in cultures of fibroblasts from lung tissues of three normal individuals (NLFb1–3) and three patients with interstitial lung disease (ILDFb1–3). Fig. 1 shows Western blots for Met, CD44v6, and collagen-1 stimulation is a major cause for up-regulation of Met and CD44v6 expression in the ILDFbs compared with NLFbs. The ratio of Met and CD44v6 expression levels of c-Met and CD44v6 mRNA was calculated after normalization with the housekeeping gene β-actin.

Statistical Analyses

Each experiment was repeated three times for each set of fibroblasts, which were considered as n = 9, and pooled for statistical analysis. Western blot analyses, mRNA analyses, and proliferation experiments for each separate experiment were repeated three or four times, depending upon the particular experiment. Data are expressed means ± S.D. Statistical analyses of the Western blots were done using Student’s t test with Mann-Whitney modification or analysis of variance as applicable. p ≤ 0.05 was considered statistically significant.

RESULTS

Synthesis of CD44v6 and c-Met (Met) Proteins in Cultured Lung Fibroblasts from ILD Patients and Normal Subjects—Protein expression levels of Met and CD44v6 were analyzed in cultures of fibroblasts from lung tissues of three normal individuals (NLFb1–3) and three patients with interstitial lung disease (ILDFb1–3). Fig. 1 shows Western blots for Met (A and B) and CD44v6 (C and D) with β-tubulin as an internal control. Cultures were treated with either Met shRNA (A and B) or CD44v6 shRNA (C and D) or with control shRNA. The ratio of Met/β tubulin was 0.95 ± 0.3 for NLFbs compared with 3.4 ± 1.3 for ILDFbs (Fig. 1E). The ratio of CD44v6/β-tubulin was very low (0.3 ± 0.08) in NLFbs and very high (6–7 ± 1.2) in ILDFbs (E). These data indicate that synthesis of Met was significantly increased and that CD44v6 was constitutively highly expressed in ILDFbs compared with NLFbs.

We hypothesized that autocrine TGF-β1 stimulation is a major cause for up-regulation of Met and CD44v6 expression in the ILDFbs. In order to show if the TGF-β1 induction of CD44 is particular to CD44v6-containing isoforms of CD44, we analyzed expression of CD44v6 and CD44s in response to TGF-β1
(10 ng/ml for 24 h). As shown in Fig. 1F, TGF-β1 selectively induced CD44v6, whereas CD44s (CD44 standard form) remained almost unchanged. In order to show if CD44v6 shRNA selectively silences all CD44v6-containing isoforms, we analyzed relative mRNA steady-state levels for CD44v6 and CD44s by qRT-PCR in RNA isolated from ILDFbs treated with TGF-β1 or transfected either with CD44v6 shRNA alone or with CD44v6 shRNA after TGF-β1 treatment. The results show that this shRNA only inhibits 1–5% of CD44s mRNA (Fig. 1G). In contrast, CD44v6-containing isoforms were strongly suppressed (Fig. 1H), which is in accordance with our previous CD44v6 shRNA results, both in vitro and in vivo (36, 37, 40, 42–44).

Localization of Met and CD44v6 in Sections of Lungs from Bleomycin- and Saline-treated Mice and in Sections of Lungs from a Normal Individual and an ILD Patient—Fig. 2 shows that Met staining is present in lung alveolar epithelia of normal mouse and normal human lung sections, whereas CD44v6 staining is relatively weak. CD44v6 staining is much stronger in areas of inflammation and fibrosis in the lung sections of the bleomycin-treated mouse and the ILD patient, with significant colocalization of Met. The increase in CD44v6 with associated Met staining was particularly striking in the degenerated lung epithelium of the ILD patient. The results in Fig. 2 suggest that induction of CD44v6 and Met proteins occurs in the accumulated lung inflammatory cells/fibroblasts at the sites of injury in the bleomycin model and the ILD patient.

FACS Sorting of Fibroblasts from Normal and ILD Lungs—We next determined the phenotype of ILDFbs that are enriched in α-SMA/collagen-1 and if they are the most active fibrotic fibroblasts that are required for fibrosis. The cells from the ILDFbs and NLFbs were sorted by FACS into CD44v6 and CD44s populations in ILDFbs. The data in these experiments (G and H) are from three sets of each lung fibroblast population with three independent experiments for each mRNA level and are expressed as the means ± S.D. Statistical analysis was done using analysis of variance as applicable. p ≤ 0.05 (*p) was considered statistically significant.
Cultures of CD44v6+/Met+ NLFbs and ILDFbs were treated with TGF-β1, and cultures of ILDFbs were also treated with TGF-β1 siRNA or control siRNA as described under “Experimental Procedures.” Fig. 4 shows 1) that TGF-β1 increases mRNA expression levels 3–4-fold for both CD44v6 (Fig. 4A) and Met (Fig. 4B) in the CD44v6+/Met+ subpopulation of the NLFbs (lanes 2 and 4 compared with lanes 1 and 3) and 1.7–1.8-fold for the ILDFbs (lanes 6 and 10 compared with lanes 5 and 9) and 2) that silencing TGF-β1 with TGF-β1 siRNA significantly reduces the endogenous high levels of CD44v6 and Met mRNAs in the CD44v6+/Met+ subpopulation of the ILDFbs (to 30–50%; lanes 8 and 12 compared with lanes 7 and 11). These results indicate that the relative basal up-regulated CD44v6 and Met mRNA expressions in ILDFbs are probably due to autocrine TGF-β1 signaling.

**CD44v6 and Met Protein Expressions Are Induced by TGF-β1—**

If autocrine TGF-β1 stimulation is the main cause for up-regulation of Met and CD44v6 expression in the ILDFbs, exogenous TGF-β1 may increase Met and CD44v6 expression in NLFbs. To address this, we first compared the levels of TGF-β1 secreted by NLFbs and ILDFbs during a time course experiment with unstimulated cultures. Fig. 5 shows that bioactive TGF-β1 secretion was extensively elevated in ILDFbs and reached nearly plateau levels by 48 h. This demonstrates directly that the autocrine production occurs significantly in ILDFbs.

Next, we investigated the effect of exogenous TGF-β1 on Met and CD44v6 expressions at the protein and mRNA levels. NLFbs were cultured to 70% confluence and then incubated for 24 h under serum starvation. The dose of TGF-β1 was first optimized for the concentration that induced the highest level of Met expression. Fig. 6A shows that TGF-β1 induced Met protein expression in a dose-dependent manner with maximum induction at 10 ng/ml, and this level remained steady...
CD44v6, Met, and TGF-β1 Signaling in Interstitial Lung Disease

Sorted CD44v6+/Met+ fractions from NLFbs and ILDFbs

![A.](image)

**FIGURE 4.** Levels of Met and CD44v6 expression in CD44v6+/Met+ FACS-sorted populations of NLFbs and ILDFbs. A and B, CD44v6+/Met+ sorted NLFbs and ILDFbs were cultured without or with 10 ng/ml TGF-β1 for 24 h and analyzed for CD44v6 mRNA and Met mRNA. CD44v6+/Met+ sorted ILDFbs were transfected with control siRNA (scrambled siRNA) or TGF-β1 siRNA and cultured for 96 h. Total RNAs were extracted from the transfected and treated cell populations and analyzed for Met and CD44v6 by qRT-PCR as described under “Experimental Procedures.” The data in these experiments (A and B) are from three sets of each lung fibroblast population with three independent experiments for each mRNA level and are expressed as the means ± S.D. (error bars). Statistical analyses were done using Student’s t test with Mann-Whitney modification as applicable. p ≤ 0.05 (*) was considered statistically significant.

![B.](image)

**FIGURE 5.** TGF-β1 synthesis by ILDFbs and NLFbs. ILDFb1–3 and NLFb1–3 cultures were incubated for different times and assayed for the bioactive form of TGF-β1 by an ELISA as described under “Experimental Procedures.” The data represent three independent experiments for each set of lung fibroblasts. Error bars, means ± S.D.

through 30 ng/ml. Therefore, 10 ng/ml TGF-β1 was used for subsequent experiments with NLFbs. The cultures were incubated for 2–16 h with or without various concentrations of TGF-β1. Fig. 6B shows that TGF-β1 induced Met protein synthesis between 4 and 8 h, which was sustained for 16 h. Further, CD44v6 protein expression was also greatly elevated at 8–16 h (Fig. 6C). Blocking endogenous TGF-β1 expression with TGF-β1 siRNA significantly diminished the expression levels of Met and CD44v6 proteins (Fig. 6D) and of mRNAs (Fig. 6E) in ILDFb1 and ILDFb3 cultures. Taken together, the data in Fig. 6, A–E, support the hypothesis that up-regulation of Met and CD44v6 protein and mRNA expression in ILDFbs involves autocrine TGF-β1 regulation.

**Cell Proliferation of ILDFbs and of TGF-β1-stimulated NLFbs Is Dependent on a Met and CD44v6 Pathway**—The possible functional relevance of the ability of TGF-β1 to induce Met and CD44v6 was first tested by analyzing cell proliferation by BrdU uptake, a measure of DNA replication and an index of cell proliferation. The BrdU uptake in Fig. 7A and the bar graph in Fig. 7B show that both NLFbs and ILDFbs treated for 24 h with TGF-β1 in the presence of a control IgG exhibited greatly increased proliferation compared with TGF-β1 treatment in the presence of a TGF-β1-blocking antibody (Fig. 7B, lanes 4 compared with lanes 5). Conversely, treatments with TGF-β1 siRNA significantly inhibited proliferation compared with a control siRNA (Fig. 7B, lanes 3 compared with lanes 2). The basal level of proliferation in ILDFb3s was ~4-fold higher compared with NLFb3s (data not shown). These results strongly suggest that autocrine TGF-β1 is required for hyperproliferation of the ILDFbs.

TGF-β1 has been shown to mediate the activation of a number of downstream targets associated with PI3K signaling, in particular ERK (45–48). Thus, to elucidate the molecular mechanisms responsible for TGF-β1-dependent cell proliferation, we first assayed the activation status of a variety of signaling molecules in ILDFbs without TGF-β1 treatment. Fig. 8, A and B (statistical analyses of replicates), shows that blocking Met with Met siRNA (lane 3 compared with lane 2) and CD44v6 with CD44v6 siRNA (lane 4 compared with lane 2) significantly inhibited basal activation of pp38MAPK, pAKT, and pERK in ILDFb3s, suggesting that endogenous TGF-β1 is one of the major factors responsible for the basal levels of their phosphorylations (49). These results show that the activations of ERK1/2, PI3K/AKT, and p38MAPK depend primarily on the function of Met and CD44v6.

Fig. 8C (lanes 2–4) also shows that these treatments significantly inhibited cell proliferation to ~50% (Met siRNA) and ~30% (CD44v6 siRNA). Further, ILDFb3 cultures were exposed to optimal concentrations of pharmacologic inhibitors that are widely used to block specific signaling pathways. Fig. 8, A (lanes 5–7) and B (statistical analyses of replicates), shows that the U0126 ERK inhibitor, the LY294002 PI3K/AKT inhibitor, and the SC68376 p38 MAPK inhibitor each significantly inhibited phosphorylation of its respective protein. Whereas inhibition of pp38MAPK was selective, inhibitors of pERK and pAKT showed cross inhibition, indicating that PI3K-dependent inhibition of ERK involves AKT. Such cross-talk between PI3K and ERK exists in differentiating epithelial cells (50). Fig. 8C also shows that down-regulation of endogenous TGF-β1 with TGF-β1 siRNA strongly inhibited cell proliferation (lane 5 compared with lane 2).
Fig. 8D (lane 3) shows that inhibition of pERK significantly decreased cell proliferation (to ~25% of control siRNA; lane 2), and that inhibition of either pAKT or pp38 (lanes 4 and 5) decreased cell proliferation to a lesser extent (to ~75% of control siRNA; lane 2). These results show that autocrine TGF-β1 strongly regulates cell proliferation via MAPK/ERK1/2 signaling (lane 3 compared with lane 2), whereas PI3K and p38MAPK pathways have less effect on cell proliferation (lanes 4 and 5). However, blockade of Met activation by Met siRNA did not further affect the U0126-mediated decrease of proliferation in ILDFb3s (Fig. 8D, lane 7 compared with lane 3). Therefore, it is unlikely that ERK signal transduction pathways have any major role in mediating Met-induced proliferation in ILDFbs. On the other hand, blockade of CD44v6 activation by CD44v6 siRNA further down-regulated the U0126-mediated decrease of proliferation (Fig. 8D, lane 6 compared with lane 3), indicating that CD44v6-mediated cell proliferation acts by an ERK-dependent pathway. It is also possible that this additional effect with CD44v6 siRNA involves another signaling pathway, whereas the Met-mediated cell proliferation is ERK-independent.

Fig. 8E shows the effects of blocking activation of ERK, PI3K/AKT, and p38MAPK on TGF-β1-induced cell proliferation in NLFb2s. Treatment with TGF-β1 (lane 5) increased cell proliferation more than 3-fold compared with NLFb2s that were not treated with exogenous TGF-β1 (lane 1). Blockade of ERK activation significantly decreased cell proliferation in untreated NLFb2s (Fig. 8E, lane 2 compared with lane 1), whereas exogenous TGF-β1 in the presence of the ERK inhibitor restored cell division to near the level with TGF-β1 alone (Fig. 8E, lane 6 compared with lane 5). Blockade of PI3K/AKT and p38 activation in untreated cultures also showed some inhibition of cell proliferation (Fig. 8E, lanes 3 and 4 compared with lane 1). However, the addition of TGF-β1 did not increase cell division when p38 activation was inhibited (Fig.
8E, lane 8 compared with lane 4), whereas inhibition of PI3K/AKT activation had only a modest increase of cell division in the presence of TGF-β1 (Fig. 8E, lane 7 compared with lane 3) relative to the increase when ERK was inhibited (Fig. 8E, lane 6).

These results are therefore unique in demonstrating that ERK, AKT, and p38 MAPK were constitutively phosphorylated in ILDFbs and that blocking Met and CD44v6 abrogated phosphorylation of p38 MAPK, AKT, and ERK1/2. Further, TGF-β1 induction of cell proliferation in ILDFbs appears to depend primarily on the function of ERK and less so on PI3K/AKT but not on p38 MAPK. Moreover, TGF-β1-induced CD44v6 mediates cell proliferation via an ERK-dependent pathway, whereas a TGF-β1-induced Met pathway is ERK-independent.

**Synthesis of HGF by NLFBs and ILDFbs**—Pulmonary fibroblasts are an important source of cytokines, growth factors, and mediators that control alveolar epithelial cell proliferation and differentiation. HGF is one of the key factors produced by fibroblasts important in lung pathology (51). In patients with acute lung injury, elevated levels of HGF are found in both serum (52, 53) and bronchoalveolar lavage fluid, suggesting its involvement in human lung repair (54). The biological influence of HGF is mediated through its specific receptor, the c-Met tyrosine kinase (55). Recent studies demonstrate that HGF is a potent antifibrotic cytokine that prevents α-SMA-positive myofibroblast activation of mesangial cells and interstitial fibroblasts, and it blocks mesenchymal transdifferentiation of tubular epithelial cells induced by TGF-β1 (28, 56–59). Many factors with positive influence on lung fibroblast activation have been described, but little is known about the molecular mechanism of HGF-induced ILDFb activation in the context of α-SMA expression. To address this, we measured HGF production during a time course experiment with unstimulated cultures of NLFBs and ILDFbs (Fig. 9). In both groups, the production of HGF increased to nearly plateau levels by 50–75 h with significantly higher levels in the ILDFbs.

**HGF Blocks Activation of α-SMA and Overproduction of Matrix Induced by TGF-β1 in CD44v6+/−Met+/− Subpopulations of ILDFbs**—Our data indicate that FACS-sorted CD44v6+/Met+ ILDFbs are the most fibrogenic subpopulations, and CD44v6−/Met− ILDFbs are comparatively less fibrogenic subpopulations, suggesting that CD44v6 and Met surface markers are major determinants for their increased synthesis of α-SMA and collagen-1 for the fibroblast activation. Thus, we examined the impact of blocking the HGF pathway on TGF-β1-mediated activation of α-SMA in these subpopulations. Fig. 10A shows
that treatment of the ILDFb1–3 CD44v6+/Met+ subpopulations for 72 h with 50 ng/ml of HGF was sufficient to inhibit synthesis of α-SMA protein. Fig. 10B shows Western blots of α-SMA for lysates of the two ILDFb1 (CD44v6+/−/Met+/−) subpopulations treated with TGF-β1 with or without HGF over a 72-h time course, and Fig. 10C shows bar graphs for replicates. TGF-β1 increased α-SMA in both subpopulations, which was significantly inhibited by the presence of HGF.

Immunoblots in Fig. 10D show that treatment of the ILDFb1–3 CD44v6+/Met+ subpopulations for 72 h with 50 ng/ml HGF was sufficient to inhibit synthesis of collagen-1. Further, qRT-PCR analyses in Fig. 10, G and H, show that the mRNA steady-state levels for the α1 and α2 chains of collagen-1 (alleles COL1A1 and COL1A2, respectively) were signif-
CD44v6, Met, and TGF-β1 Signaling in Interstitial Lung Disease

FIGURE 11. HGF-mediated reduction in α-SMA expression in ILDFbs is through an ERK1/2 pathway. In all of the experiments, every 12 h, the media were changed with fresh media containing freshly added HGF (50 ng/ml) and/or TGF-β1 (10 ng/ml). A, stimulation of the CD44v6+/Met+ subpopulation of ILDFb1s with HGF for 30 min results in activation of pAKT, pERK1/2, and pp38, as shown by Western blots (WB) with phospho-specific antibodies. Total AKT, ERK1/2, and p38 protein levels were also assessed as controls for protein loading. B, CD44v6+/Met+ ILDFb1s were pretreated with or without vehicle (DMSO) or with U0126, LY294002, or SC68376 for 30 min. The cells were washed after treatment with inhibitors for 30 min and then treated with HGF for 72 h followed by TGF-β1 for 72 h. α-SMA expression was analyzed by Western blots. Results shown are representative of three independent experiments. C, α-SMA protein levels for the different treatments in B were quantitated by scanning densitometry and corrected for the levels of β-tubulin from three sets of ILDFbs. Data (as means ± S.D. (error bars)) are expressed relative to untreated samples (100 arbitrary units). D, ILDFb2 cells were transfected with HGF expression plasmids, the transfectants were allowed to grow for 48 or 72 h. The total protein was extracted, and Western blots were probed for Smad7 protein and β-actin. Results are representative of three independent experiments. E, ILDFb2 cells were treated with 10 ng/ml TGF-β1 for 3 or 24 h, or they were first transfected with HGF expression plasmids, and the transfectants were allowed to grow for 72 h followed by treatment with 10 ng/ml TGF-β1 for 3 h. Nuclear extracts were prepared (see “Experimental Procedures”), and Western blots were probed for phosphorylated Smad2 protein and β-actin. Results are representative of three independent experiments.

significantly increased in the ILDFb1–3 CD44v6+/Met+ subpopulations compared with sorted cultures (Fig. 10, G and H, lanes 2 compared with lanes 1) and that the increases in collagen-1 protein expression by ILDFb1–3 were inhibited by the presence of HGF (Fig. 10D). In addition, CD44v6+/Met+ subpopulations have 40–45% lower mRNA expression of collagen-1 α1 (Fig. 10G) and α2 (Fig. 10H) compared with CD44v6+/Met+ subpopulations (lanes 3 compared with lanes 2). Further, in CD44v6+/Met+ subpopulations, HGF antagonizes TGF-β1-induced activation of collagen-1 protein (Fig. 10E) and mRNA expression (Fig. 10I). However, HGF is less effective in CD44v6+/Met+ subpopulations in reducing TGF-β1-induced collagen-1 protein (Fig. 10, F compared with E) and mRNA expression (Fig. 10, J compared with I). Thus, the results in Fig. 10 indicate that primarily Met, and CD44v6 in part, are important determinants for collagen matrix production. Importantly, HGF, when combined with Met, antagonizes TGF-β1-induced activation of collagen matrix production efficiently in the CD44v6+/Met+ subpopulation and abrogates enhancement of α-SMA in response to TGF-β1. The observed decrease in the amount of these collagenas in the CD44v6+/Met+ subpopulation suggests that both CD44v6 and Met overproduction by autocrine TGF-β1 signaling regulate collagen matrix production necessary for the activation of ILDFbs.

Interference with TGF-β1 Signaling Down-regulates the Expression of α-SMA—Although considerable attention has been given to the effects of HGF in mesangial cells, it remains unclear whether HGF has similar effects on interstitial lung injuries. We next investigated the signaling pathways that are important for HGF inhibition of TGF-β1-mediated α-SMA expression in the ILDFb1 CD44v6+/Met+ subpopulation. Fig. 11A shows that this subpopulation treated with HGF for 30 min, as described under “Experimental Procedures,” significantly activated pAKT and the MAPK family members pERK1/2 and pp38. Fig. 11, B and C, shows the effects of blocking activation of these signaling pathways on synthesis of α-SMA in this subpopulation treated with HGF without or with TGF-β1 as described under “Experimental Procedures.” Treatment with HGF alone inhibited α-SMA expression (lane 2 compared with lane 1). This inhibition was blocked by U0126, the MAPK/ERK1/2 inhibitor (lane 3), but not by LY294002, the PI3k/AKT inhibitor, or by SC68376, the p38 inhibitor (lanes 4 and 5). HGF completely inhibited the increased α-SMA expression induced by TGF-β1 (lane 7 compared with lane 6), and like treatments with HGF alone, the ERK inhibitor restored the TGF-β1 increase of α-SMA expression (lane 8), whereas the PI3k/AKT and p38 inhibitors did not (lanes 9 and 10). These results indicate that HGF reduces α-SMA protein in an ERK/MAPK-dependent manner in Met+ ILDFb1s, which is independent of CD44v6 and occurs in the presence or absence of TGF-β1. They also indicate that the TGF-β1 induction of α-SMA protein in these cells probably involves p38 and PI3K/AKT activation because inhibitors of these pathways almost completely inhibited TGF-β1-induced α-SMA expression.

Recent research suggests that HGF expression plasmids can repress renal injury (29, 60–63). HGF has been reported to intercept Smad2/3 translocation to the nucleus in intestinal fibroblasts (56) and in lung epithelial cells (64). HGF also specifically antagonizes the profibrotic action of TGF-β1 in mesangial cells by up-regulating Smad transcriptional corepressor TGFβ and increases expression of the transcriptional
corepressor SnoN in tubular epithelial cells (28). However, the mechanism of HGF-induced suppression of α-SMA expression in ILDFbs is not clear. To explore whether HGF intersects the TGF-β1 signaling pathway in ILDFbs, we assessed Smad7 protein expression in these cells. To achieve relatively stable expression of HGF, ILDFb2 cells were transfected with HGF expression plasmids, total protein was extracted, and Western blots were probed for Smad7 protein expression in these cells. To achieve relatively stable expression of HGF, ILDFb2 cells were transfected with HGF expression plasmids, total protein was extracted, and Western blots were probed for Smad7 protein. Smad7 levels began to increase at 48 h after HGF overexpression and remained elevated for 72 h (Fig. 11D). To determine if HGF-dependent Smad7 induction involved activation of TGF-β1 downstream signaling, we examined the effects of HGF on Smad2 phosphorylation, a critical step in the TGF-β1-mediated signal transduction pathway. As shown in Fig. 11E, TGF-β1 induced Smad2 phosphorylation significantly at 3 h after TGF-β1 stimulation and remained elevated through 24 h. However, pretreatment with HGF did not affect Smad2 nuclear translocation (Fig. 11E).

CD44v6 Regulates Lung Fibroblast Activation by Increasing Matrix Deposition in Response to TGF-β1—We examined the effects of HGF on TGF-β1-induced CD44v6 expression in the CD44v6+/Met+ subpopulation of ILDFbs. Fig. 12A shows that HGF increased the TGF-β1-induced CD44v6 expression in these cells (lanes 6–9 compared with lanes 2–5), indicating that sustained production of CD44v6 in response to HGF and TGF-β1 might influence profibrotic behavior of ILDFbs. In order to address this issue, we examined the relationship of CD44v6 with TGF-β receptors by immunoprecipitation of CD44 followed by immunoblot analysis of TGF-β receptors, TGF-βRI and TGF-βRII, and by immunoprecipitation of TGF-βRI, followed by immunoblot analysis of CD44v6. Fig. 12B shows that immunoprecipitates of CD44v6 contained both TGF-βRI and TGF-βRII, and immunoprecipitates of TGF-βRI contained CD44v6. This suggests that these TGF-β receptors associate with CD44v6 in ILDFbs.

We then examined whether the sustained production of CD44v6 in response to TGF-β1 can affect matrix production. Fig. 12C shows that silencing CD44v6 suppresses TGF-β1 increases of collagen-1 and its chaperone, HSP47, in NLFbs (lanes 3 compared with lane 2). Further, silencing either CD44v6 or TGF-β1 in untreated ILDFbs also inhibited collagen-1 production (lanes 6 and 7 compared with lane 5). The results in Figs. 9–12 indicate that the CD44v6+/Met+ subpopulations in ILDFbs contain the most active fibrogenic fibroblasts that augment α-SMA and collagen-1 (α1 and α2) necessary for ILD.

The association between CD44v6 and collagen-1 expression was further examined by immunocytochemistry and fluorescent microscopy in lung tissue sections from bleomycin- and saline-treated mice (Fig. 13) and in a lung section from an ILD patient compared with a control lung section (Fig. 14). Immunocytochemistry using a CD44v6 monoclonal antibody and a collagen-1 polyclonal antibody showed significant co-localization (yellow) in regions of inflammation/fibrosis in sections of lungs from the bleomycin-treated mouse and to some extent in the ILD patient. In contrast, CD44v6 and collagen-1 staining were much less in the sections of lungs from the saline-treated mouse and the normal control.
CD44v6, Met, and TGF-β1 Signaling in Interstitial Lung Disease

FIGURE 13. Localization of CD44v6 (red), collagen-1 (green), and nuclei (DAPI) (blue) in sections of lungs from mice treated with bleomycin or saline. Representative micrographs show Masson staining for collagen expression (top panels) and for localization of collagen-1 (green), CD44v6 (red), and nuclei (blue) in the lung sections of mice 21 days after treatment with bleomycin (middle panels) or saline (bottom panels). The micrographs are representative of sections from three mice in each group. The images in each panel are at the same magnification (scale bars, 100 μm).

FIGURE 14. Localization of CD44v6 (red), collagen 1 (green), and nuclei (DAPI) (blue) in sections of lungs from a normal individual and an ILD patient. Representative micrographs show Masson staining for collagen expression (top panels) and for localization of collagen-1 (green), CD44v6 (red), and nuclei (blue) in the lung sections from the ILD patient (middle panels) or the normal subject (bottom panels). The micrographs are representative of sections from three subjects in each group. The images in each panel are at the same magnification (scale bars, 100 μm).

DISCUSSION

The importance of pathological changes leading to progressive lung dysfunction in the interstitium of ILD is now well recognized. With increasing awareness of the importance of these pathological interstitial changes, interest has focused on the role of myofibroblasts in the initiation and progression of a fibrotic response. In fibrotic lungs, the phenotypic transformation of a majority of myofibroblasts from the quiescent to the activated state is accompanied by accumulation of extracellular matrix, α-SMA, and TGF-β1, which is a hallmark in most, if not all, types of fibrosis, including ILD observed in animal models and humans (3, 4, 9, 65–67). Emerging studies implicate TGF-β1 as a profibrotic factor and HGF/Met signaling as an antifibrotic pathway in the tissue fibrosis. Other important players related to TGF-β1 are HA and its receptor CD44, particularly CD44v6, as shown in this study.

Previous studies from our laboratory demonstrated that HA interaction with CD44v6 modulates cellular responses to growth factor/cytokines (36) and that the resulting signaling is activated by both ligand-dependent and -independent receptor tyrosine kinases in epithelial cells (34, 35, 39, 40). Whereas CD44 and HA are overexpressed in human and animal inflammatory diseases (68, 69), the endogenous binding of HA to CD44v6 is more avid than to standard CD44 (CD44s) in these pathological conditions (40, 44, 68–75). Although increased expression of HA receptor CD44 has been documented in both acute injury (76) and progressive fibrosis (23), its role in the pathogenesis of these disease processes is not clear. Nevertheless, the therapeutic targets that can be manipulated to control the development of fibrotic disorders have not been studied well, and the mechanisms that drive these disorders are inadequately determined. Thus, the aim of the current study was to define the relationship between CD44v6 and TGF-β1 and the potential modulating influence of HGF on TGF-β1-dependent CD44v6 signaling and function in ILD fibroblasts.

In this report, we have demonstrated that the expression of HGF/Met was spontaneously induced in cultured fibroblasts derived from ILD patients. In particular, constitutive expression of Met protein and gene in fibroblasts was observed not only in patients with ILD but also to a certain extent in NLFbs (Fig. 1). Seemingly contradictory results have been reported on the specific expression of Met and HGF in SSC dermal fibroblasts but not in normal fibroblasts (77, 78), which may simply reflect differences in cell types studied and experimental approaches used. Interestingly, high constitutive expression of CD44v6 was observed in ILDFbs, whereas much lower expression was detected in NLFbs (Fig. 1, A–F). Our observations suggest that CD44v6 and Met expression appear at the same time frame after TGF-β1 induction in NLFbs and ILDFbs (Fig. 6), indicating that CD44v6 and Met probably have an important role in the control of the fibrotic phenotype of ILDFbs. This possibility was supported by results in Fig. 4, A and B, showing that silencing TGF-β1 significantly decreased Met and CD44v6 in Western blots. The mRNA analysis (Fig. 4, A and B) revealed that TGF-β1 increased the expression of both CD44v6 and c-Met mRNAs in the CD44v6+/Met+ FACS-sorted fraction in NLFbs and ILDFbs, and the ILDFbs produce much more CD44v6 and Met compared with NLFbs when stimulated by TGF-β1, indicating further that the basal up-regulated CD44v6 and Met expressions in ILDFbs (Figs. 3E and 4 [A and B]) may be due to autocrine TGF-β1 signaling. Additionally, the pattern of colocalization of CD44v6 and Met expression in the flow cytometry appears to be tightly correlated with TGF-β1 induction in the sorted CD44v6+/Met+ populations of NLFbs and ILDFbs (Figs. 3 and 4). Similarly, the colocalization of CD44v6 and Met was evident in the lung sections from fibrotic lungs of bleomycin-treated mice and patients with ILD, and the expressions of these two proteins were markedly higher in the fibrotic lung sections compared with their corresponding normal sections (Fig. 2, A and B).
Furthermore, ELISAs of culture supernatants showed increased secretion of TGF-β1 in ILDFbs compared with NLFs (Fig. 5). Immunoblotting analyses revealed that TGF-β1 increased the expression of Met in NLFs in a dose- and time-dependent manner (Fig. 6, A and B) with a coordinated time increase of CD44v6 (Fig. 6C). Conversely, ILDFbs treated with the TGF-β1 siRNA to silence autocrine TGF-β1 signaling showed a marked reduction in the protein and mRNA expressions of CD44v6 and Met (Fig. 6, D and E). Strong immunofluorescent colocalization of CD44v6 and Met receptors was observed in regions of inflammation/fibrosis of lung sections from a bleomycin-treated mouse and an ILD patient (Fig. 2), suggesting that their induction occurs in lung epithelial cells and myofibroblasts at sites of injury. Therefore, the up-regulated Met and CD44v6 expressions in ILDFbs may be due to autocrine TGF-β1 signaling. This possibility was further supported by the augmented level of TGF-β1 in ILDFbs (Fig. 5).

The overexpression of Met may be associated with the increased expression of MMP-1 mediated by HGF, which has been shown previously to have a role in the antifibrotic behavior of the HGF/Met pathway in ILDFbs (1, 25–27). On the other hand, the overexpression of CD44v6 may be a fibrogenic feedback response to the fibrosis in ILDFbs. These biological feedback responses may also occur under other fibrotic mechanisms. Interestingly, the TGF-β1/Met-induced replication of ILDFb cells does not depend on an ERK pathway (Fig. 7, D and E) but may be associated with other mechanisms, such as TGF-β1-Smad signaling, which needs to be investigated in future studies. In contrast, replication of ILDFbs induced by TGF-β1/CD44v6 primarily depends on MAPK pathways (Fig. 7, D and E), which is supported by the activation of major signaling molecules pERK, pAKT, and pp\(38^{MAPK}\) (Fig. 7, B and C).

Such close sequential and coordinated association between CD44v6 and Met in response to TGF-β1 in Figs. 1–7 prompted us to investigate a potential role of HGF in TGF-β1-induced ILDFb activation and matrix production by using CD44v6 \(-/-\)/Met \(-/-\) FACS-sorted populations as an in vitro model system. Of note, a previous report demonstrated increased Met expression in SSc dermal fibroblasts (77, 79), although neither the HGF/Met signaling mechanisms involved nor the effect of HGF on CD44v6 up-regulation by endogenous TGF-β1 were addressed, and the role for how HGF/Met counteracts the antifibrotic effect of HGF needs to be addressed more clearly. Importantly, there has been no report that discusses the role of the modulating influence of HGF on TGF-β1-induced CD44v6-dependent signaling and function in ILDFbs.

In our investigations of the involvement of ERK pathways in the HGF-mediated decrease in α-SMA levels and participation of ERK pathways in fibroblast proliferation, it was evident that ERK has different roles in mediating HGF effects. Now the question is how ERK signaling contributes to HGF effects. Numerous reports indicate the involvement of ERK in the inhibition of TGF-β signaling. It is possible that our finding that HGF induced Smad7 in ILDFbs may be associated with ERK activation, or HGF-induced Smad7 may inhibit α-SMA. In line with these possibilities, MEK inhibition blocks Smad7 protein induction in rat epithelial cells (64). Although our studies show that HGF induced by Smad7 does not involve Smad phosphor-

ylation and nuclear translocation after HGF treatment (Fig. 10, D and E), the inhibitory action of HGF on TGF-β1 signaling is unlikely to operate at a nuclear level. Such findings are consistent with the observation demonstrating that HGF blocks mesangial activation while not affecting Smad2 activation (28). Our studies indicate that HGF-induced Smad7 may involve alternate mechanisms (i.e. Smad7 may antagonize other transcription factors, such as c-Myc or TGFβ, that mediate α-SMA transcriptional down-regulation) (28). Our observation that Met-induced fibroblast proliferation does not require ERK indicates that HGF-induced \(38^{MAPK}\) (Fig. 11A) may be involved in HGF/Met-induced cell proliferation. In addition, during repeated injury in fibrosis, the dominant expression of TGF-β1 over HGF is associated with the progression of chronic fibrosis (62). Thus, with repeated injury, TGF-β1 and TGF-β1-induced CD44v6 will stimulate ILDFb proliferation. These findings indicate that the ratio of TGF-β1 and HGF is closely associated with the pathogenesis and progression of chronic ILD fibrosis.

Because one of the consequences of autocrine TGF-β1 signaling in ILDFb activation is to overproduce CD44v6 (Figs. 2–6), we examined the effects of HGF on TGF-β1-mediated induction of CD44v6 expression in CD44v6 \(-/-\)/Met \(+/-\) populations of ILDFbs (Fig. 12). We propose that Met overexpression through autocrine TGF-β1 signaling is more sensitive to the effects of HGF. The contradiction that increased Met expression coincided with increased collagen-1 expression in ILDFbs may be explained by the finding that HGF levels in these fibrotic disorders were much lower (as shown in Fig. 8) than those used in the experiments (Fig. 10), suggesting that the increase in serum HGF levels may be insufficient to regulate collagen synthesis in ILDFbs. This possibility is consistent with the inhibitory effect of higher concentrations (50–100 ng/ml) of HGF on α-SMA expression and on collagen-1 protein and mRNA expressions in CD44v6 \(-/-\)/Met \(+/-\) subpopulations of ILDFbs, indicating that the antifibrotic effects of HGF primarily require a high level of HGF in ILDFbs, and this action is through Met. As shown in Figs. 6 and 12, TGF-β1 induced both Met and CD44v6 expression in CD44v6 \(-/-\)/Met \(+/-\) populations of ILDFbs, and HGF effectively abolished TGF-β1 activation and TGF-β1-induced collagen-1 in Met \(+/-\) fibroblasts (Fig. 10, C–F). CD44v6 \(-/-\)/Met \(+/-\) ILDFbs produce less collagen-1 protein and mRNA expression, and the inhibitory effect of HGF on the collagen-1 signal is less effective in this subpopulation of cells (Fig. 10, E–F). This indicates that primarily Met, and CD44v6 in part, are responsible for TGF-β1-regulated collagen-1. Importantly, HGF further induced TGF-β1-induced CD44v6 expression in these cells (Fig. 12).

Our study on the contribution of MAPK pathways in the HGF-mediated decrease to TGF-β1-induced α-SMA expression levels shows that ERK, P38/\(38^{MAPK}\), and p38\(^{MAPK}\) have different roles in mediating HGF effects. Whereas ERK was required for the inhibitory effects of HGF, HGF-induced p38\(^{MAPK}\) and P38/\(38^{MAPK}\) promoted α-SMA expression levels. Therefore, it is possible that although HGF has an antifibrotic effect, HGF levels may be insufficient to prevent overproduction of matrix, and HGF can still increase α-SMA levels through the activation of p38\(^{MAPK}\) and P38/\(38^{MAPK}\) (Fig. 11, A and B).

Our results in Fig. 11C demonstrated that the association of CD44v6 and TGF-β1 receptors facilitated the attenuation of
FIGURE 15. Model for involvement of CD44v6 and Met due to autocrine TGF-β1 signaling in ILDFbs. The elevated HGF expression at the onset of chronic injury may compensate and support a regenerative process (62), but repetitive lung injury results in overexpression of TGF-β1 and TGF-β1-induced autocrine signaling that induces a sustained expression of CD44v6 and Met that activates ILDFbs with subsequent increased collagen matrix synthesis. In normal lung fibroblasts, TGF-β1 treatment also activates the Met and CD44v6 receptors. Although HGF interferes with TGF-β1 signaling, in view of the fact that HGF decreases in a reciprocal manner to the increase in TGF-β1 level (for reference, see “Conclusion”) during the progression of chronic injury in ILD fibrosis, TGF-β1-induced CD44v6 and Met can have a crucial role for the sustained ILDFb fibrogenic activation.

ILDFb response to TGF-β1-induced collagen-1 synthesis. More specifically, we have demonstrated a decrease in synthesis of collagen-1 in response to blockage of CD44v6 by CD44v6 siRNA in ILDFbs when cells were stimulated with HGF in the presence of TGF-β1. These results (Figs. 10 and 12) emphasize that HGF and CD44v6 trigger distinct signal transduction pathways for inhibition and promotion of fibrosis, respectively. Given that a reciprocal balance between CD44v6 and HGF/Met can regulate the pathology of chronic ILDFbs, how this reciprocal balance is achieved deserves special investigation.

Thus, the initial activation of TGF-β1 after lung injury could be favorable for promoting a wound-healing process because HGF can counteract TGF-β1-induced function. However, it is reasonable to predict that a sustained exposure to hyperactive TGF-β1 by its autocrine signaling will ultimately move the system in favor of profibrotic effects through sustained CD44v6 signaling. In accordance with this, the TGF-β1 autocrine signal to induce CD44v6 is greatly amplified in the presence of HGF despite the antifibrotic action of the HGF/Met pathway (see the hypothetical model in Fig. 15).

CONCLUSION

In summary, in response to acute lung injury, HGF increases in the blood circulation derived from non-injured organs and injured organs (62, 80–82). Our findings indicate that HGF levels in ILDFbs increased from normal lung fibroblasts (Fig. 9). These findings are in accordance with the observed serum levels of HGF that are markedly increased in several fibrotic disorders, including SSc (83, 84). These contradictions may be explained by the finding that HGF levels in these fibrotic fibroblasts were much lower than those used to examine the effect of the exogenous addition of HGF on suppression of collagen-1 and α-SMA expression in our study (Figs. 10, 11, and 12A), suggesting that the increase in ILDFbs of HGF levels is insufficient to regulate collagen-1 matrix synthesis in ILD fibroblasts and their activation. These findings indicate that during progression of chronic lung fibrosis, levels of HGF decrease, as has been seen in other organ fibrosis, where TGF-β1 strongly suppresses expression of HGF mRNA (85–87). The exogenous HGF-mediated suppression of TGF-β1 signaling (Fig. 11) in ILDFbs may be directly through the Met targeting cellular processes that are essential for fibrogenesis. Furthermore, our studies indicate that TGF-β1 autocrine signaling in ILDFbs induces a sustained expression of CD44v6 that activates cell cycle progression via ERK activation and increased collagen matrix synthesis. Taken together, it is reasonable to propose that a persistent, chronic exposure to hyperactive TGF-β1 signaling that sustains CD44v6 will eventually overwhelm the system in favor of profibrotic effects of TGF-β1 (Fig. 15). Therefore, the balance between TGF-β1 and HGF appears to have a critical role in determining whether the injured tissues undergo recovery or fibrogenesis.

Acknowledgments—We thank Nathan J. Gibson and Margaret H. Romano (Medical University of South Carolina) for providing the protocol for immunohistochemistry.

REFERENCES

1. Kajihara, I., Jinnin, M., Makino, T., Masuguchi, S., Sakai, K., Fukushima, S., Maruo, K., Inoue, Y., and Ihn, H. (2012) Overexpression of hepatocyte growth factor receptor in scleroderma dermal fibroblasts is caused by autocrine transforming growth factor β signaling. Biosci. Trends 6, 136–142
2. Schnaper, H. W., Hayashida, T., Hubchak, S. C., and Poncelet, A. C. (2003) TGF-β signal transduction and mesangial cell fibrogenesis. Am. J. Physiol. Renal Physiol. 284, F243–F252
3. Ziyadeh, F. N., Hoffman, B. B., Han, D. C., Iglesias-De La Cruz, M. C., Hong, S. W., Isono, M., Chen, S., McGowan, T. A., and Sharma, K. (2000) Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial expansion by treatment with monoclonal antithrombinantigrrowth factor β antibody in db/db diabetic mice. Proc. Natl. Acad. Sci. U.S.A. 97, 8015–8020
4. Han, D. C., Hoffman, B. B., Hong, S. W., Guo, J., and Ziyadeh, F. N. (2000) Therapy with antisense TGF-β1 oligodeoxynucleotides reduces kidney weight and matrix mRNAs in diabetic mice. Am. J. Physiol. Renal Physiol. 278, F528–F534
5. Ihn, H., Yamane, K., Kubo, M., and Tamaki, K. (2001) Blockade of endogenous transforming growth factor β signaling prevents up-regulated collagen synthesis in scleroderma fibroblasts. Association with increased expression of transforming growth factor β receptors. Arthritis Rheum. 44, 474–480
6. Kikuchi, K., Hirtl, C. W., Smith, E. A., LeRoy, E. C., and Trojanowska, M. (1992) Direct demonstration of transcriptional activation of collagen gene expression in systemic sclerosis fibroblasts. Insensitivity to TGF β1 stimulation. Biochem. Biophys. Res. Commun. 187, 45–50
7. Jinnin, M., Ihn, H., Mimura, Y., Asano, Y., and Tamaki, K. (2006) Potential regulatory elements of the constitutive up-regulated α2(I) collagegn gene in scleroderma dermal fibroblasts. Biochem. Biophys. Res. Commun. 343, 904–909
8. Lanka, V. N., and Phan, S. H. (2006) The extrapulmonary origin of fibroblasts. Stem/progenitor cells and beyond. Proc. Am. Thorac. Soc. 3, 373–376
9. Hinz, B., Phan, S. H., Thannickal, V. J., Galli, A., Bochaton-Piallat, M. L., and Gabbiani, G. (2007) The myofibroblast. One function, multiple origins. Am. J. Pathol. 170, 1807–1816
10. Phan, S. H. (2003) Fibroblast phenotypes in pulmonary fibrosis. Am. J. Respir. Cell Mol. Biol. 29, 587–592
11. Desmoulière, A., Darby, I. A., and Gabbiani, G. (2003) Normal and pathologic soft tissue remodeling. Role of the myofibroblast, with special emphasis on liver and kidney fibrosis. Lab. Invest. 83, 1689–1707
12. Levesque, H., Baudot, N., Delpech, B., Vayssairat, M., Gancel, A., Lauret, P., and Courtois, H. (1991) Clinical correlations and prognosis based on hyaluronic acid serum levels in patients with progressive systemic sclerosis. Br. J. Dermatol. 124, 423–428
13. Freitas, J. P., Filipe, P., Emerit, I., Meunier, P., Manso, C. F., and Guerra Rodrigo, F. (1996) Hyaluronic acid in progressive systemic sclerosis. Der-
CD44v6, Met, and TGF-β1 Signaling in Interstitial Lung Disease

14. Foster, L. C., Arkonac, B. M., Sibinga, N. E., Shi, C., Perrella, M. A., and Bjermer, L., Engstrom-Laurent, A., Lundgren, R., Rosenhall, L., and Hallberg, L. (1990) CD44 is the principal cell surface receptor for hyaluronate. *J. Biol. Chem.* 265, 1303–1313

15. Jinnin, M., Ihn, H., Mimura, Y., Asano, Y., Yamane, K., and Tamaki, K. (2005) Effects of hepatocyte growth factor in lung fibroblasts from white scleroderma patients via two directional cell motility. *J. Biol. Chem.* 280, 20310–20315

16. Misra, S., Ghatkar, S., Zoltan-Jones, A., and Toole, B. P. (2003) Regulation of multidrug resistance in cancer cells by hyaluronan. *J. Biol. Chem.* 278, 25285–25288

17. Misra, S., Ghatkar, S., and Toole, B. P. (2005) Regulation of MDRI expression and drug resistance by a positive feedback loop involving hyaluronan, phosphoinositide 3-kinase, and ErbB2. *J. Biol. Chem.* 280, 20310–20315

18. Misra, S., Ghatkar, S., De Giovanni, C., Markwald, R. R., and Ghatkar, S. (2009) Delivery of CD44 shRNA/nanoparticles within cancer cells. Perturbation of hyaluronan/CD44v6 interactions and reduction in adeno gene in ApC Min/+ MICE. *J. Biol. Chem.* 284, 12432–12446

19. He, S., Liu, X., Yang, Y., Huang, W., Xu, S., Yang, S., Zhang, X., and Robb, M. J. (1996) CD44v6, Met, and TGF-β1 couples Ras activation and CD44 alternative splicing. *Genes Dev.* 20, 1715–1720

20. Misra, S., Ghatkar, S., and Toole, B. P. (2006) A positive feedback loop couples Ras activation and CD44 alternative splicing. *Genes Dev.* 20, 1715–1720

21. Mucsi, I., Skorecki, K. L., and Goldberg, H. J. (1996) Extracellular signal-regulated kinase and the small GTP-binding protein, Rac, contribute to the effects of transforming growth factor-β1 on gene expression. *J. Biol. Chem.* 271, 7863–7868

22. Atlı, A., Dijeloul, S., Chastre, E., Davis, R., and Geppert, C. (1997) Evidence for a role of Rho-like GTPases and stress-activated protein kinase c-Jun N-terminal kinase (SAPK/JNK) in transforming growth factor β-mediated signaling. *J. Biol. Chem.* 272, 1429–1432

23. Bakin, A. V., Tomlinson, A. M., Bhowmik, N. A., Moses, H. L., and Arteaga, C. L. (2000) Phosphatidylinositol 3-kinase function is required for transforming growth factor β-mediated epithelial to mesenchymal transition and cell migration. *J. Biol. Chem.* 275, 36803–36810

24. Bhowmik, N. A., Ghiasi, M., Bakin, A., Aakre, M., Lundquist, C. A., Engel, M. E., Arteaga, C. L., and Moses, H. L. (2001) Transforming growth factor-β mediates epithelial to mesenchymal differentiation through a RhoA-dependent mechanism. *Mol. Biol. Cell.* 12, 27–36

25. He, S., Liu, X., Yang, Y., Huang, W., Xu, S., Yang, S., Zhang, X., and Robberts, M. S. (2010) Mechanisms of transforming growth factor β1/Smad signalling mediated by mitogen-activated protein kinase pathways in keratinocytes. *Br. J. Dermatol.* 162, 538–546

26. Lapiere, P., Langlois, M. J., Boucher, M. J., Jobin, C., and Rivard, N. (2004) Down-regulation of MEK/ERK signaling by E-cadherin-dependent PI3K/Akt pathway in differentiating intestinal epithelial cells. *J. Cell. Physiol.* 199, 32–39

27. Ware, L. B., and Matthey, M. A. (2002) Keratinocyte and hepatocyte...
growth factors in the lung. Roles in lung development, inflammation, and repair. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **282**, L1924–L1940.

52. Maeda, J., Ueki, N., Hada, T., and Higashino, K. (1995) Elevated serum hepatocyte growth factor/scatter factor levels in inflammatory lung disease. *Am. J. Respir. Crit. Care Med.* **152**, 1587–1591.

53. Yamanouchi, H., Fujita, J., Yoshinouchi, T., Hojo, S., Kamei, T., Yamadori, I., Ohtsuki, Y., Ueda, N., and Takahara, J. (1998) Measurement of hepatocyte growth factor in serum and bronchoalveolar lavage fluid in patients with pulmonary fibrosis. *Respir. Med.* **92**, 273–278.

54. Stern, J. B., Fierobe, L., Paugam, C., Rolland, C., Dehoux, M., Petiet, A., Dombret, M. C., Mantz, J., Aubier, M., and Crestani, B. (2000) Keratinocyte growth factor and hepatocyte growth factor in bronchoalveolar lavage fluid in acute respiratory distress syndrome patients. *Crit. Care Med.* **28**, 2326–2333.

55. Giordano, S., Ponzetto, C., Di Renzo, C. F., Cooper, C. S., and Comoglio, P. M. (1989) Tyrosine kinase receptor indistinguishable from the c-met protein. *Nature* **339**, 155–156.

56. Yang, J., Dai, C., and Liu, Y. (2003) Hepatocyte growth factor suppresses renal interstitial myofibroblast activation and intercepts Smad signal transduction. *Am. J. Pathol.* **163**, 621–632.

57. Liu, Y. (2004) Epithelial to mesenchymal transition in renal fibrogenesis. Pathologic significance, molecular mechanism, and therapeutic intervention. *J. Am. Soc. Nephrol.* **15**, 1–12.

58. Inoue, T., Okada, H., Kobayashi, T., Watanabe, Y., Kanno, Y., Kopp, J. B., Nishida, T., Takigawa, M., Ueno, M., Nakamura, T., and Suzuki, H. (2003) Hepatocyte growth factor counteracts transforming growth factor-β1, through attenuation of connective tissue growth factor induction, and prevents renal fibrogenesis in 5/6 nephrectomized mice. *FASEB J.* **17**, 268–270.

59. Yang, J., and Liu, Y. (2002) Blockade of tubular epithelial to myofibroblast transition by hepatocyte growth factor prevents renal interstitial fibrosis. *J. Am. Soc. Nephrol.* **13**, 96–107.

60. Ono, I., Yamashita, T., Hida, T., Jin, H. Y., Ito, Y., Hamada, H., Akasaka, Y., Ishii, T., and Jimbow, K. (2004) Local administration of hepatocyte growth factor gene enhances the regeneration of dermis in acute incisional wounds. *J. Surg. Res.* **120**, 47–55.

61. Matsumoto, K., Mizuno, S., and Nakamura, T. (2000) Hepatocyte growth factor in renal regeneration, renal disease and potential therapeutics. *Curr. Opin. Nephrol. Hypertens.* **9**, 395–402.

62. Matsumoto, K., and Nakamura, T. (2001) Hepatocyte growth factor. Renotropic role and potential therapeutics for renal diseases. *Kidney Int.* **59**, 2023–2038.

63. Mizuno, S., Matsumoto, K., and Nakamura, T. (2001) Hepatocyte growth factor suppresses interstitial fibrosis in a mouse model of obstructive nephropathy. *Kidney Int.* **59**, 1304–1314.

64. Shukla, M. N., Rose, J. L., Ray, R., Lathrop, K. L., Ray, A., and Ray, P. (2009) Hepatocyte growth factor inhibits epithelial to myofibroblast transition in lung cells via Smad. *Am. J. Respir. Cell Mol. Biol.* **40**, 643–653.

65. Forbes, S. J., Russo, F. P., Rey, V., Burra, P., Rugge, M., Wright, N. A., and Rugge, M. (2006) Matrix metalloproteinase-1 up-regulation by hepatocyte growth factor in human dermal fibroblasts via ERK signaling pathway involves Ets1 and Fli1. *Nucleic Acids Res.* **35**, 3540–3549.

66. Takada, S., Namiki, M., Takahara, S., Matsumiya, K., Kondoh, N., Kokado, Y., Morimatsu, H., Saitoh, Y., Ito, T., Tsuda, H., Matsuda, T., Matsuoka, Y., Kondo, H., Saitoh, Y., Imamura, H., and Hirohashi, S. (2001) c-MET expression in myofibroblasts. Role in autocrine activation and prognostic significance in lung adenocarcinoma. *Am. J. Pathol.* **158**, 1451–1463.

67. Innin, M., Ibhn, M., Mimura, Y., Asano, Y., Yamane, K., and Tamaki, M. (2005) Matrix metalloproteinase-1 up-regulation by hepatocyte growth factor in human dermal fibroblasts via ERK signaling pathway involves Ets1 and Fli1. *Nucleic Acids Res.* **33**, 3540–3549.

68. Yang, I., Nagaika, M., Ishibashi, H., Niho, Y., Matsumoto, K., and Nakamura, T. (1992) Lung may have an endocrine function producing hepatocyte growth factor in response to injury of distal organs. *Biochem. Biophys. Res. Commun.* **182**, 802–809.

69. Ono, K., Matsumori, A., Shioli, T., Furukawa, Y., and Sasayama, S. (1997) Enhanced expression of hepatocyte growth factor/c-Met by myocardial ischemia and reperfusion in a rat model. *Circulation* **95**, 2552–2558.

70. Miyazawa, K., Shimomura, T., Nakamura, T., and Kitamura, N. (1994) Proteolytic activation of hepatocyte growth factor in response to tissue injury. *J. Biol. Chem.* **269**, 8966–8970.

71. Kawaguchi, Y., Harigai, M., Hara, M., Fukasawa, C., Takagi, K., Tanaka, M., Tanaka, E., Nishimaga, E., and Kamatani, N. (2002) Expression of hepatocyte growth factor and its receptor (c-met) in skin fibroblasts from patients with systemic sclerosis. *J. Rheumatol.* **29**, 1877–1883.

72. Yamanouchi, H., Fujita, J., Yoshinouchi, T., Hojo, S., Kamei, T., Yamadori, I., Ohtsuki, Y., Ueda, N., and Takahara, J. (1998) Measurement of hepatocyte growth factor in serum and bronchoalveolar lavage fluid in patients with pulmonary fibrosis. *Respir. Med.* **92**, 273–278.

73. Wielenga, V. J., van der Voort, R., Mulder, J. W., Kruyt, P. M., Weidema, W. F., Oosting, J., Seldenrijk, C. A., van Krimpen, C., Offerhaus, G. J., and Pals, S. T. (1998) CD44 splice variants in human colon cancer is related to tumor progression. *Cancer Res.* **58**, 4754–4756.