Lung fibroblasts accelerate wound closure in human alveolar epithelial cells through hepatocyte growth factor/c-Met signaling

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Lung fibroblasts accelerate wound closure in human alveolar epithelial cells through hepatocyte growth factor/c-Met signaling. Am J Physiol Lung Cell Mol Physiol 307: L94–L105, 2014.—There are 190,600 first published April 18, 2014; doi:10.1152/ajplung.00233.2013.—Jewish Health, Dept. of Medicine, Smith Bldg., A448, 1400 Jackson St., ACUTE LUNG INJURY/ACUTE RESPIRATORY DISTRESS SYNDROME (ALI/ARDS) each year in the United States, and the incidence and mortality of ALI/ARDS increase dramatically with age. Patients with ALI/ARDS have alveolar epithelial injury, which may be worsened by high-pressure mechanical ventilation. Alveolar type II (ATII) cells are the progenitor cells for the alveolar epithelium and are required to reestablish the alveolar epithelium during the recovery process from ALI/ARDS. Lung fibroblasts (FBs) migrate and proliferate early after lung injury and likely are an important source of growth factors for epithelial repair. However, how lung FBs affect epithelial wound healing in the human adult lung has not been investigated in detail. Hepatocyte growth factor (HGF) is known to be released mainly from FBs and to stimulate both migration and proliferation of primary rat ATII cells. HGF is also increased in lung tissue, bronchoalveolar lavage fluid, and serum in patients with ALI/ARDS. Therefore, we hypothesized that HGF secreted by FBs would enhance wound closure in alveolar epithelial cells (AECs). Wound closure was measured using a scratch wound-healing assay in primary human AEC monolayers and in a coculture system with FBs. We found that wound closure was accelerated by FBs mainly through HGF/c-Met signaling. HGF also restored impaired wound healing in AECs from the elderly subjects and after exposure to cyclic stretch. We conclude that HGF is the critical factor released from FBs to close wounds in human AEC monolayers and suggest that HGF is a potential strategy for hastening alveolar repair in patients with ALI/ARDS.

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ACUTE LUNG INJURY/ACUTE RESPIRATORY DISTRESS SYNDROME (ALI/ARDS) is a severe form of diffuse lung disease that imposes a substantial health burden all over the world. The incidence and mortality of ALI/ARDS increase with age (50, 52). ALI/ARDS is associated with alveolar epithelial injury and increased epithelial permeability resulting in alveolar edema, severe hypoxemia, and a decrease in lung compliance, which usually requires intubation and mechanical ventilation. Mechanical ventilation is a life-saving supportive measure because it allows use of positive end expiratory pressure and allows delivery of high levels of inspired oxygen. However, extensive animal data have demonstrated that it also can contribute to lung injury in a process termed ventilator-induced lung injury (VILI) (52). Although many approaches have been undertaken to improve the outcome of ARDS pharmacologically, the results have been disappointing. One area that has not received a great deal of attention is a focus on epithelial repair.

The alveolar epithelium is comprised of two cell types, alveolar type I (ATI) cells and alveolar type II (ATII) cells. ATI cells cover about 95% of the alveolar surface and participate in transepithelial fluid movement and innate immunity but are sensitive to injury. ATII cells produce pulmonary surfactant and also participate in transepithelial fluid movement and innate immunity but are more resistant to injury, perhaps because of their limited apical surface exposure to the alveolus and their high-volume density of organelles (55). ATII cells are thought to be the main progenitor cells for restoring the alveolar epithelium after injury (3, 12, 15). However, recently other progenitor cells have also been shown to be important after catastrophic injury to type II cells (4, 30, 59). After epithelial injury in the skin, the cornea, the trachea, or the lung, the initial response is for the surviving epithelial cells at the edge of the wound to dedifferentiate, spread, migrate, then proliferate, and finally redifferentiate to restore the epithelium (20, 28, 38, 65).

During this process the epithelial cells may receive signals from underlying mesenchymal cells. Mesenchymal cells are critical for epithelial development in the fetal lung, and lipofibroblasts are thought to be important in the repair of the alveolar epithelium in adult animals (3). Fibroblasts (FBs) have been shown to control proliferation and differentiation of alveolar epithelial cells (AECs) through direct contact (39, 53), as well as by secretion of soluble mediators such as hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) (44, 60, 63).

In this report, we studied wound repair in primary human AECs and how lung FBs accelerate epithelial wound healing. We hypothesized that HGF secreted by lung FBs would promote wound closure in human AECs. HGF, originally named scatter factor, is thought to be important in lung injury (41) and is known to stimulate the migration of epithelial cells (16, 47, 62). In addition, we examined whether wound closure by human AECs differed with age and/or mechanical stretch, which mimics the mechanical ventilation in vivo. Age is well known to be an important prognosis factor in ARDS but has not been studied in detail. This research contributes to our understanding of how lung FBs augment alveolar epithelial repair and the importance of HGF for accelerating wound repair by human AECs.

MATERIALS AND METHODS

Donor information. To isolate human primary ATII cells, alveolar macrophages (AMs), and FBs, we obtained human lungs from de-identified organ donors whose lungs were not suitable for transplantation and donated for medical research through the National Disease
Research Interchange (Philadelphia, PA) and the International Institute for the Advancement of Medicine (Edison, NJ), The Committee for the Protection of Human Subjects at National Jewish Health deemed this research as not human subject research. We selected donors with reasonable lung function with a PaO2/FIO2 ratio of >225, no history of clinical lung disease, a chest radiograph that indicated no infection, and a time on the ventilator of less than 5 days. The sex, age, race, and smoking history were variable and were not selection criteria. For the aging study, we defined young donors as below 40 yr of age and aged donors as over 65 yr of age, and we used cells only from nonsmokers.

**Isolation and culture of human ATII cells.** We modified the human type II cell isolation method published by Fang and coworkers (13, 58). Briefly, the middle lobe was perfused, lavaged, and then instilled with elastase (Worthington, Lakewood, NJ) for 40 min at 37°C. The lung was minced, and the cells were isolated by filtration and partially purified by centrifugation on a discontinuous density gradient made of Optiprep (Accurate Chemical Scientific, Westbury, NY) with densities of 1.080 and 1.040 and by positive selection with MACS MicroBeads human CD326 (EpCAM) (Miltenyi Biotech, Bergisch Gladbach, Germany). Cell preparations were made to assess cell purity by staining for cytokeratin (CAM 5.2; Dako Cytomation, Carpenteria, CA) and surfactant protein A (antibody; a gift from Yoshio Kuroki, Sapporo, Japan). The isolated cells were resuspended in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 2.5 μg/ml amphotericin B, 100 μg/ml streptomycin, 100 μg/ml penicillin G (GIBCO, Life Technologies, Rockville, MD), and 10 μg/ml gentamicin (Sigma-Aldrich, St. Louis, MO). One million cells per square centimeter were plated on either 12- or 24-well cell culture plates (Costar, Corning, NY) that had been previously coated with rat-tail collagen (RTC) (in triplicate for the control) or 500 ng/ml anti-human HGF antibody (R&D Systems, Minneapolis, MN).

**Quantitative measurement of 40 human growth factors using culture medium from human lung FBs.** Five million FBs were plated on a 100-mm tissue-culture dish in DMEM with 10% FBS. When the cells became confluent, they were washed once and cultured with DMEM without serum overnight. The following day, the cells were incubated either with or without 100 nM adenosine 5'[−y-thio] triphosphate tetralithium salt (ATP−y-S) (Sigma), 10 ng/ml recombinant human IL-1α, or IL-1β (R&D Systems). After 48 h, we collected the culture media from the four different conditions (control, ATP−y-S, IL-1α, and IL-1β), which were stored at −80°C. We measured different human growth factors in these samples using the Quantibody Human Growth Factor Array 1 (RayBiotech, Norcross, GA). Briefly, this array used a multiplexed sandwich ELISA-based technology. The target growth factors were trapped on the glass surface by capturing antibodies, then biotin-labeled detection antibodies were added, which recognized the target growth factors, and finally the growth factor-antibody-biotin complex was visualized through the addition of the streptavidin-labeled Cy3 equivalent dye and measured with a laser scanner (Agilent Technologies G2505B Micro Array Scanner; Agilent Technologies, Santa Clara, CA). After the detection, we analyzed the data with the Human Growth Factor Q-Analyzer (RayBiotech) as recommended by the supplier.

**Wound closure assay in AEC monolayer with recombinant human growth factors.** As described above, when cultured AECs formed a monolayer, the cells were washed and cultured overnight in DMEM without serum. The following day, we made crisscross scratch wounds in the AEC monolayers made on wells in either 24-well or 12-well plates. To compare the effect of growth factors on wound closure in AEC monolayers, we added 10 ng/ml of each recombinant human growth factor in DMEM without serum to the scratched AEC monolayers, took phase-contrast pictures of wounds in AEC monolayers under the microscope at 0 and 24 h after wounding and adding growth factors, measured wound areas using Image J software, and analyzed the degree of wound closure based on the area of control condition as 1.00. Human recombinant HGF and KGF were obtained from Amgen (Thousand Oaks, CA), and human recombinant basic FB growth factor (bFGF), FGF4, growth differentiation factor (GDF)-15, insulin growth factor (IGF)-1, bone morphogenetic protein 5 (BMP-5), osteoprotegrin (OPG), and vascular endothelial growth factor (VEGF) were purchased from R&D Systems.

**Real-time RT-PCR.** For real-time RT-PCR, the expression levels of genes were expressed as a ratio to the expression of the constitutive probe Cyclophilin B. The specific primers and probes for HGF were purchased from Applied Biosystems (Foster City, CA).
**HGF ELISA assay and Western blotting.** To measure the concentration of HGF in the culture medium, we used a human HGF ELISA (R&D Systems) according to the instruction manual.

For the Western blotting analysis, polyacrylamide gradient gels (8–16%; Invitrogen) were run in Tris glycine buffer to separate the proteins. Proteins were run in the reduced state. Protein loading was normalized to GAPDH. The primary antibodies were anti-Met (c-Met) antibody (Abcam, Cambridge, MA), anti-phospho-Met (Cell Signaling Technology, Danvers, MA), anti-phospho-focal adhesion kinase (FAK) (Y397) (Life Technologies, Grand Island, NY), anti-phospho-Src (Tyr416) (Cell Signaling Technology), anti-phospho-ERK (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-JNK (Abcam), and anti-phospho-Akt (Cell Signaling Technology).

**Cell motility assay.** Human ATII cells were plated and cultured on RTC-coated 35-mm glass-bottom dishes (MatTek, Ashland, MA) until the AECs formed a monolayer. Crosssection wounds were made with p10 pipette tips and cultured with or without 50 ng/ml HGF. Cells were then mounted on a culture dish heater (DH-35; Warner Instruments, Hamden, CT) fitted with a TC-344B dual automatic temperature controller (Warner Instruments). Cells were imaged at 37°C on an inverted microscope (Zeiss Axiovert 200M; Carl Zeiss, Jena, Germany) using a ×63 oil-immersion lens. Images were acquired and analyzed using Intelligent Imaging Innovations 3D software. Time-lapse images (exposure 500 ms) were taken every 20 min. For each series, 54 images were taken. To quantitate cell motility, over 30 cells were randomly chosen, the center of the nucleus was used as a reference point, and travel distance was measured for 18 h. The cell motility was then expressed as the distance the cell moved in microns in 18 h (26).

**Immunofluorescence staining and measurement of lamellipodia area.** For immunofluorescence microscopy, cells were fixed with 4% paraformaldehyde and then permeabilized in phosphate-buffered saline containing 0.2% Triton X-100. Cells were stained with phalloidin-tetramethylrhodamine B isothiocyanate (Sigma), anti-vinculin antibody (Sigma), and DAPI (Vector Laboratories, Burlingame, CA) by standard immunofluorescence procedures and imaged with an inverted Zeiss Axiovert 200M deconvolution microscope (Carl Zeiss). Images were acquired and processed using Slidebook 5.0 software (Intelligent Imaging Innovations, Denver, CO). Lamellipodia areas were analyzed from leading edge at the four corners of crosssection wounds using Slidebook 5.0 software by tracing the shape of lamellipodia stained by phallolidin-tetramethylrhodamine B isothiocyanate.

**Cyclic stretch experiment.** We plated primary human ATII cells at a density of 4.0 million cells/well on BioFlex Collagen Type I-coated culture plates for the cyclic stretch (CS) system (Flexcell strain unit Flex Central FX-400 tension plus; Flexcell International, Hillsborough, NC). After the AEC monolayer was formed, cells were preincubated with or without 50 ng/ml HGF for 30 min, and wounds were made with P10 pipette tips. The plates were then placed on the Flexcell tension system (10% elongation, 6 cycles/min). To examine the effect of CS and HGF on wound closure, phase-contrast pictures were taken at 0 and 48 h after wounding.

**Statistical analysis.** All data are presented as means ± SE. One-way ANOVA was used to compare the difference between two or more groups. The post hoc Bonferroni/Dunnett test was used for multiple comparisons. Statistical significance was set at $P < 0.05$.

**RESULTS**

**FBs promote wound closure in human primary AEC monolayers.** To investigate whether FBs and/or AMs accelerated wound closure in scratched wounds in AEC monolayers, primary human ATII cells were plated on 12-well culture plates coated with rat tail collagen. When a confluent monolayer was visible, scratch wounds were made with p10 pipette tips, and wound closure was observed with or without FBs or AMs cultured on the 12-well inserts (Fig. 1A). FBs accelerated wound closure in AEC monolayers 1.73 times the extent compared with cultures without FBs ($P < 0.0001$) at 24 h after wounding (Fig. 1B). However, inserts containing AMs did not alter the extent of wound closure.

**Screening for growth factors released by human primary FBs.** To examine which growth factors secreted by FBs were responsible for the accelerated wound closure in AECs, we ran the Quantibody Human Growth Factor Array 1 (Raybiotech) to screen for 40 growth factors. We used medium from FBs derived from two individuals cultured with or without ATP-γ-S, IL-1α, or IL-1β. Previous reports indicated that scratch wounded releasing ATP and that the ATP analog ATP-γ-S induced rapid and sustained EGFR activation dependent on human bronchial EGF shedding and enhanced wound closure in epithelial cell lines (64). IL-1β has been reported to be the main bronchoalveolar lavage fluid (BALF) mediator involved in HGF secretion from lung FBs (46). In this experiment, we were determining which growth factors were produced by primary human lung FBs, and we found that either control or stimulated FBs secreted measurable levels of bFGF, BMP-5, FGF-4, KGF (FGF7), GDF-15, HGF, IGF-1, OPN, IGF binding proteins, and VEGF. IL-1α induced BMP-5, HGF, and IGF-1, whereas ATP-γ-S did not consistently stimulate any growth factors (data not shown).

**HGF promotes wound closure in human AEC monolayers.** To determine which growth factor/factors released by the FBs accelerate wound closure in AEC monolayers, we added 10 ng/ml recombinant human bFGF, BMP-5, FGF-4, KGF, GDF-15, HGF, IGF-1, OPN, VEGF to the scratch wounds in human primary AEC monolayer and compared the extent of wound closure to controls. We excluded IGF binding proteins from this experiment because they are binding proteins for IGFs. We tested these growth factors at a concentration of 10 ng/ml according to published papers (1, 22, 25, 27, 29, 42, 51). Of the nine growth factors tested, only HGF accelerated wound closure in AEC monolayers, which was 1.96 times the extent compared with controls without any growth factors at 24 h after wounding ($P < 0.0001$) (Fig. 2). In other experiments, we evaluated FGFl0, which was not on the Quantibody Human Growth Factor Array 1 (RayBiotech), but has been shown to be secreted by FBs and known to be a mitogen for rat ATII cells (14, 63). Neither of these factors stimulated wound closure in human AEC monolayers. These results suggest that HGF was the major factor secreted by FBs that accelerated wound closure in AEC monolayers.

**FBs promote wound repair in AECs through HGF/c-Met signaling.** To verify that the HGF/c-Met signaling pathway was responsible for the enhanced wound closure in AECs by FBs, we added 500 nM PHA 665752, a potent, selective inhibitor of Met kinase, (TOCRIS Bioscience), and 500 ng/ml of a neutralizing antibody to HGF (R&D Systems) to the FB coculture system with scratched wounds in AECs. PHA 665752 and the anti-HGF antibody diminished the accelerating effect of FBs on wound closure in AEC monolayers at 24 h after wounding (1.079 ± 0.047 $P < 0.0001$ vs. wound + FBs, 1.035 ± 0.055 $P < 0.0001$ vs. wound + FBs, respectively) (Fig. 3A). Because we observed that recombinant HGF also activated EGFR in human AEC monolayers (unpublished data), we added 100 nM AG1478, a potent and highly selective inhibitor of EGFR receptor kinase, to scratch wounds in AEC monolayers.
monolayers cocultured with FBs. However, AG1478 did not affect the accelerated wound closure by FBs (1.387 ± 0.100 P = 0.1360 vs. wound + FBs) (Fig. 3A). HGF has been reported to be expressed by rodent AMs and FBs. To better define potential sources of HGF in the human lung, we measured mRNA levels and protein levels of HGF from human AECs, AMs, FBs, and medium from coculture systems. The mRNA levels of HGF were detected only in FBs and not in AECs or AMs (Fig. 3B). The protein levels of HGF in the medium were significantly higher in coculture systems with FBs compared with cultures with AMs or without either AMs or FBs (control, 55 ± 31 pg/ml; wound with AMs, 20 ± 10 pg/ml; wound with FBs, 5,470 ± 1,380 pg/ml; wound with FBs + PHA 665752, 6,060 ± 2,400 pg/ml; wound with FBs + AG 1478, 7,680 ± 3,860 pg/ml) (Fig. 3C). We also confirmed c-Met phosphorylation in the wounded AEC monolayer at 6 and 24 h after coculturing with FBs, which was inhibited by PHA 665752 (Fig. 3D). Hence, these results suggest that FBs promote wound closure in AECs through HGF/c-Met signaling.

Additionally, we compared HGF secretion by FBs between coculture with unwounded AEC monolayers and wounded AEC monolayers. However, we did not observe a difference between these two conditions, which indicates that wounding...
HGF promotes lamellipodia formation and activates FAK, ERK1/2, Akt, and JNK, but not Src. To define how HGF promoted cell motility, we compared the areas of lamellipodia with and without HGF at 2 h after wounding. The areas of lamellipodia at the four corners of crisscross scratch wounds were significantly increased by HGF [HGF (-), 977 ± 119 μm²; HGF (+), 1,388 ± 142 μm², P = 0.0340 from 4 independent experiments] (Fig. 5, A and B). We also measured phosphorylation of the cell migration regulatory kinases FAK and Src by western blotting. FAK was activated by HGF at 30 min, whereas Src was not (Fig. 5C). We also showed the activation of other signaling kinases downstream from c-Met. FAK, Akt, ERK1/2, and JNK were all activated by HGF at 30 min from all donors (Fig. 5C). We also examined whether wounding alone would activate c-Met and these downstream kinases. We made multiple wounds in the AEC monolayers, but wounding alone did not activate c-Met, FAK, Src, Akt, ERK1/2, or JNK in our system.

HGF restores the delayed wound closure due to age and CS. Alveolar epithelial injury is a critical feature of ALI/ARDS, and recovery requires alveolar epithelial repair. The incidence and mortality of ALI/ARDS increase dramatically with age, and high-pressure mechanical ventilation is almost uniformly required for treatment of ARDS. Therefore, we sought to determine whether the wound closure in the AEC monolayer was delayed with age and/or CS and whether HGF restored the delay. Primary human ATII cells from young donors (20–39 yr old, n = 4) and aged donors (over 65 yr old, n = 4) were plated on 12-well culture plates coated with RTC. When a complete monolayer was visible, scratch wounds were made with P10 pipette tips, and wound closure was observed with or without HGF. The wound closure in AEC monolayers from aged donors was significantly delayed and was restored by HGF (young without HGF, 1.00 ± 0.04; young with HGF, 1.22 ± 0.06 P = 0.006 vs. young without HGF; elderly without HGF, 0.66 ± 0.07 P < 0.0001 vs. young without HGF; elderly with HGF, 1.00 ± 0.07 P = 0.0002 vs. elderly without HGF) (Fig. 6, A and B). Lamellipodia formation was also significantly decreased in the elderly [young 977 ± 119 μm² and elderly 457 ± 48 μm², P = 0.0003 young donors (n = 4) and elderly donors (n = 4)] (Fig. 6, C and D). However, we were not able to demonstrate that HGF enhanced the lamellipodia formation in AEC monolayers from older organ donors (Fig. 6D). To determine the effect of CS on wound closure in AEC monolayer, primary human ATII cells were plated on BioFlex Collagen Type I-coated culture plates for CS system (Flexcell tension system). When a complete monolayer was visible, 50 ng/ml HGF was added 30 min before wounding and starting CS, scratch wounds were made with P10 pipette tips, and then the cell culture plates were put on the CS system (10% elongation, 6 cycles/min). At 48 h after wounding and starting CS, CS impaired wound closure and HGF restored the impairment (no CS without HGF, 1.00 ± 0.04; no CS with HGF, 1.60 ± 0.08 P < 0.0001 vs. no CS without HGF; CS without HGF, 0.37 ± 0.10 P < 0.0001 vs. no CS without HGF; CS with HGF, 0.94 ± 0.09 P < 0.0002 vs. CS without HGF) (Fig. 7, A and B).

DISCUSSION

Using a monolayer scratch assay with human AECs, we demonstrated that lung FBs accelerated wound closure through the HGF/c-Met pathway and that HGF induced cell motility in AEC monolayers. We also found that wound closure in human...
AEC monolayer was delayed in the elderly and by CS. In both of these conditions, wound closure was accelerated by HGF.

The confluent monolayer scratch assay used in this study provides an in vitro model of cell migration, which can be used to identify factors that may be important in the in vivo setting. However, in vivo wound healing is much more complex and involves coagulation, granulation, inflammation, reepithelialization, tissue remodeling, and the interaction of many other cell types. Although any in vitro model will not be able to address all these processes simultaneously, they do allow specific factors to be investigated with individual cell types in a controlled environment (1, 16, 28, 47). We used monolayers of primary human ATII cells plated on RTC-coated plastic wells. The phenotype of these AECs may be similar to what is found in an injured lung, as these cells have lost some surfactant protein expression, acquired some type I cell markers, and morphologically became flatter and more spread but still contain residual lamellar bodies (58). ATII cells are the most susceptible to injury, presumably attributable to their large surface area and relative paucity of cellular organelles. ATII cells are responsible for covering the denuded alveolar surface and restoring the epithelium (15, 33, 56). Our study is the first in vitro wound-healing assay using primary human AECs.

Epithelial-mesenchymal interactions are important for normal lung growth, development, and the restoration of normal lung architecture after lung injury (39, 53). Proliferation and/or constitutive activation of prosurvival signaling in mesenchymal cells at the early stage of ARDS are associated with poor prognosis of patients with ARDS, which leads to fibrosis (5, 21, 32). On the other hand, epithelial growth and survival factors such as HGF and KGF (FGF-7) (44) found in BALF 21, 32). On the other hand, epithelial growth and survival factors such as HGF and KGF (FGF-7) (44) found in BALF.
healing (46, 54, 57). Lung FB migration and proliferation occur early after lung injury and are suggested to be important in lung healing. In this study, we have shown that lung FBs promote wound closure in human primary AECs and that HGF is the critical factor for the wound repair in this system. A surprising finding was that among all the factors tested, only HGF significantly accelerated the wound closure in human AEC monolayers. Other factors, such as bFGF, BMP-5, FGF-4, KGF, FGF-10, GDF-15, IGF-1, OPG, and VEGF, which are known to be associated with cell proliferation, wound closure, and/or inflammation in the cells from the different organs, had no apparent effect on wound closure (1, 19, 22, 25, 27, 29, 42, 51). These soluble factors are likely important in other organs or species, which could have different levels of receptors and receptor activation. In addition to finding high levels of HGF in the FB-conditioned media, we also confirm the importance of the HGF/c-Met pathway in this system by using phospho-c-Met inhibitor, PHA 665752, and an antibody to human HGF. Recently, Myerburg et al. (39) also showed that FBs promoted the closure of mechanically scraped wounds in human bronchial epithelial (HBE) cells through HGF secreted by FBs, which supports our conclusions (39). However, as shown in a previous report (35), we cannot rule out the possibility that activation and release of HGF from the extracellular matrix by proteases enhance wound closure in AEC monolayers. Hence, this consideration may be important in vivo. In addition, in the malignancy, epithelial cells might be a source of HGF and could be stimulated by a paracrine factor released by FBs. However, under our experimental conditions, we did not observe any HGF secretion by AECs, and they did not express the mRNA for HGF (Fig. 3B).

We previously reported that coculturing rat AMs with rat ATII cells stimulated DNA synthesis in the epithelial cells (31). However, in our current study, we observed that human AMs did not alter wound closure in a human AEC monolayer in our cocultures. A likely reason for this difference is because HGF mRNA and protein were not detected in human AMs, whereas HGF mRNA is expressed in rat AMs (34). Hence we believe that there is a significant species difference in expression of HGF in AMs.
The HGF/c-Met pathway plays important roles in the embryonic development of the liver and the placenta, in the migration of myogenic precursor cells, and in epithelial morphogenesis (40). HGF is a mitogen for rat ATII cells in vitro (34), and HGF stimulates both proliferation and migration of immortalized rat ATII cells (SV40-T2 cells) (18). HGF increases cell migration but not proliferation during human nasal epithelial repair in vitro (66). These results suggest that epithelial cells from different species and locations display different responses to HGF (9). Because the effect of HGF on primary human AECs has not been reported, in this study, we examined whether human primary AECs, which were in the middle of transdifferentiation from ATII to ATI-like cells, could migrate and/or proliferate during the wound closure. We have found that HGF enhances cell motility but not proliferation at the wound edge at 24 h and 48 h after wounding. However, HGF may stimulate proliferation at later time points.

A complex interplay between the actin cytoskeleton and cell adhesion sites is required for the generation of membrane protrusions such as lamellipodia (7, 37). Focal adhesion sites are complex and include structural proteins such as talin, paxillin, and vinculin and signaling proteins such as Src and FAK. HGF increases phosphorylation of Src and FAK and enhanced expression level of vinculin in the human prostate epithelial cell line PNT1A (45). Activation of HGF receptor (c-Met) in epithelial cells also results in lamellipodia promotion, spreading, migration, and tubular formation through ERK, FAK, and Rac activation (23). We found that lamellipodia formation was enhanced by HGF in young subjects and were able to demonstrate increased phosphorylation of FAK and ERK. However, we did not observe activation of Src (Fig. 5C) and did not observe enhanced expression of vinculin (data not shown) by HGF. Therefore, HGF is likely to enhance lamellipodia formation in the human AEC monolayer through FAK and ERK, but the details of how HGF enhances lamellipodia formation in human AEC monolayers remain to be determined.

The incidence rates and mortality of ALI/ARDS increase dramatically with age (50, 52), and animal models of ALI showed higher mortality and increased severity with age (24, ...
Although the importance of the ATII cells as progenitor cells for restoring the alveolar epithelium after alveolar damage is well documented, alterations of ATII cell function with age have not been studied. In this study, we showed that wound closure was delayed and the size of lamellipodia was decreased in AECs from elderly subjects. We also observed less peripheral distribution of vinculin in human AECs from the elderly (Fig. 6B). Arnesen et al. (2) demonstrated that Achilles tendon FBs from old mice have lower motility and proliferation, a disorganized actin cytoskeleton, and a different localization of key focal adhesion proteins such as FAK, talin, and paxillin compared with the same cells from young mice. In addition, FBs from the old mice had increased expression of an endoplasmic reticulum (ER) stress marker, GADD153 (2). Aged satellite cells, which carry out repair and maintenance of skeletal muscle as a resident stem cell population, migrate at less than half the speed of young cells (6). In this report, although HGF increased the rate of aged satellite cell movement, it did not alter...
the size of blebs on old satellite cells, which are similar to lamellipodia. Therefore, these reports support our results in human AECs. However, we did not observe increased ER stress markers such as GADD153, Bip, and ATF6, decreased phosphorylation of FAK and Src, or downregulated protein levels of vinculin and talin from whole cell lysates in the elderly (data not shown). Additional studies will be needed to define the mechanism of delayed cell motility in the elderly and how HGF restores the delayed wound repair without enhancement of lamellipodia formation. We observed cell sheet movement to close wound in AECs when we measured cell travel distance (cell motility) by time-lapse imaging. Fenteany et al. (17) have shown two distinct mechanisms to account for wound closure in epithelial cell sheets. One mechanism of epithelial sheet wound closure is the protrusion of filopodia and ruffling lamellae, which occurs at the edge of wound (the first row of cells at the wound edge), resembling the crawling behavior of the free cells. Another mechanism is the contribution of cells behind the wound margin, which are called submarginal cells, push in, and close the wound. Thus, although we could not observe the detectable change in the size of lamellipodia by HGF in the elderly, HGF might activate submarginal cells, move cells deeper in the monolayer, and lead to the restoration of the delayed wound closure in the elderly.

Mechanical ventilation in ALI/ARDS is lifesaving but also can contribute to lung injury in a process termed VILI (43, 52). Previously, Crosby et al. (8,9) reported that mechanical stretch by CS slowed wound repair and caused apoptosis and gap formation in rat AEC monolayers. KGF has been known to accelerate wound closure in airway epithelial cell lines during cyclic mechanical strain (61). In our study, we have confirmed that CS delayed wound closure and that HGF restored the delay in human AEC monolayers. Previously, ATII cells isolated from rats exposed to hyperoxia and high-tidal-volume mechanical ventilation exhibited significantly decreased cell adhesion and reduced phosphorylation of FAK and the protein levels of paxillin compared with control rats (10). CS also decreased cell migration in human airway cell line 16HBE by inhibiting PI3 kinase and FAK-mediated JNK activation (11). Although wound closure was delayed by CS, we did not observe decreased phosphorylation of FAK and Src or decreased protein

Fig. 7. HGF restores the delayed wound closure due to cyclic stretch (CS). A: phase-contrast pictures were taken from marked wound areas at 0 and 48 h after wounding to compare the degree of wound closure among 4 different groups (wounded AEC monolayers without HGF, with HGF, on CS without HGF, and on CS with HGF). B: degree of wound closure was analyzed at 48 h after wounding. Values are means ± SE for 4 independent experiments. *P < 0.05.
levels of vinculin and talin by CS (data not shown). One reason for this variance could be because we used a lower level of CS (10%, 6 cycle/min) compared with the previous report (20%, 30 cycle/min), as higher levels of CS caused our cells to detach from the membrane.

In summary, we have shown that FBs enhanced wound closure in the human AEC monolayer through the HGF/c-Met pathway and that HGF promoted wound closure by accelerating cell motility but not proliferation. We have also found that wound closure was delayed with age and CS, which were restored by HGF. These findings suggest that HGF would be a potential treatment for repairing the denuded areas of alveolar epithelium during acute lung injury. Additional studies are required to determine the mechanism of how HGF enhances the wound closure in the elderly and during CS.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: Y.I., K.C., and J.A.S. performed experiments; Y.I. and K.C. analyzed research; Y.I., K.C., and J.A.S. interpreted results of experiments; Y.I., K.C., and J.A.S. contributed to writing of the paper; and R.J.M. edited and revised manuscript.

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