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Coordinate expression of fibulin-5/DANCE and elastin during lung injury repair

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Kuang, Ping-Ping, Ronald H. Goldstein, Yue Liu, David C. Rishikof, Jyh-Chang Jean, and Martin Joyce-Brady. Coordinate expression of fibulin-5/DANCE and elastin during lung injury repair. *Am J Physiol Lung Cell Mol Physiol* 285: L1147–L1152, 2003. First published August 8, 2003; 10.1152/ajplung.00098.2003.—Fibulin-5, previously known as DANCE and EVEC, is a secreted extracellular matrix protein that functions as a scaffold for elastin fiber assembly and as a ligand for integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_9\beta_1$. Fibulin-5 is developmentally regulated in the lung, and lung air space enlargement develops in mice deficient in fibulin-5. Fibulin-5 is also induced in adult lung following lung injury by hyperoxia. To further examine the role of fibulin-5 during repair of lung injury, we assessed fibulin-5 expression during elastase-induced emphysema in C57/b mice. Mice were treated with either saline or elastase via the trachea, and the lung was examined 20 days after treatment. Fibulin-5 mRNA was induced almost fourfold, whereas elastin mRNA was minimally elevated. Immunohistochemistry studies showed that fibulin-5 was induced in cells within the alveolar wall following elastase treatment. Western analysis demonstrates that fibulin-5 was strongly expressed in isolated primary lung interstitial fibroblasts. Fibulin-5 protein was localized to the fibroblast cell layer in culture, and brief elastase treatment degraded the protein. Intact fibulin-5 did not accumulate in the culture media. Treatment of fibroblasts with the proinflammatory cytokine interleukin-1β abolished fibulin-5 mRNA expression. Our results indicate that fibulin-5 is coordinately expressed and regulated with elastin in lung fibroblasts and may serve a key role during lung injury and repair.

developmental arteries and neural crest epidermal growth factor-like; elastase; interferon-γ; interleukin-1β

THE FIBULIN-5/DANCE GENE IS expressed in elastin-enriched tissue and plays a key role during assembly of tropoelastin into elastin fibers by linking extracellular elastin with the cell surface. This linkage occurs via multiple EGF repeats in fibulin-5 that bind elastin in a calcium-dependent fashion, and an arginine-glycine-aspartate (RGD) motif in the first fibulin-5 EGF domain that binds to cell surface integrins (5, 12). This bridging function may be required for proper elastic fiber organization as the fibulin-5 null phenotype exhibits severe elastinopathy in the skin, the vasculature, and the lung (11, 19). We showed previously that fibulin-5 gene expression is developmentally regulated from the late fetal through the postnatal period in the lung. The predominant cellular site of fibulin-5 expression was the vascular endothelial cell as assessed by in situ hybridization. However, fibulin-5 mRNA expression was also evident in lung interstitial cells, albeit at a much lower level (4).

Lung interstitial elastin is derived from interstitial fibroblasts during both postnatal development and repair following injury. We reported that elastin mRNA expression, as assessed by in situ hybridization, was upregulated in lung tissue, including interstitial cells, in a rodent model of elastase-induced emphysema (9). The cytokine IL-1β inhibits elastase expression by lung interstitial fibroblasts, suggesting that a proinflammatory milieu impairs the process of lung repair following injury (6, 8). To confirm this hypothesis, we showed that the severity of elastase-induced emphysema was highly reduced in mice lacking both IL-1β and TNF-α receptors as well as mice lacking solely the IL-1β receptor, although to a lesser degree (8). These results suggest that inflammatory mediators, including IL-1β, amplify the air space enlargement resulting from elastolytic injury. Others have shown that another cytokine, interferon (IFN)-γ, also induces emphysema in mice when overexpressed in the lung, and the mechanism involves inhibition of connective tissue formation (18). Because fibulin-5 is intimately associated with elastin and also produced during lung injury repair, we hypothesized that lung interstitial fibroblasts would express and regulate the fibulin-5 gene in a coordinate fashion with elastin.

METHODS

Materials. Electrophoresis supplies for RNA analysis were obtained from International Biotechnologies (New Haven, CT) and for protein analysis from Bio-Rad (Rockville Center, NY). The BenchMark Prestained Protein Ladder from Invitrogen (Carlsbad, CA) was used as the standard for protein electrophoresis. General chemical supplies were from Sigma (St. Louis, MO). Rat fibulin-5, elastin, and β-actin cDNA probes were previously described by Jean et al. (4) and Kuang et al. (6). The affinity-purified rabbit antibody (BSYN 1923) against the rat fibulin-5 peptide YRGPYSNPYSTSYS-

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GPYPAAAPP was the generous gift of Drs. H. Yanagisawa and E. N. Olson (University of Texas Southwestern Medical Center, Dallas, TX). This peptide spans *residues 76–98* in the intact protein and is conserved between mouse, rat, and human fibulin-5 (19). A polyclonal antibody against α-tubulin was purchased from Sigma.

Model. For whole animal study, C57/b mice (Charles River Breeding Laboratory, Wilmington, MA) were exposed to porcine pancreatic elastase (PPE) as described previously by Lucey et al. (8). The lungs were examined after 20 days, given that this previous work demonstrated an effect of IL-1β on the remodeling process at this time. Procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee at Boston University School of Medicine. For cell studies, fibroblasts were isolated from the lungs of 8-day-old Sprague-Dawley rats and maintained in culture (6). In some experiments, confluent cultures of cells were treated with IL-1β (250 pg/ml) and IFN-γ (200 pg/ml) separately and in combination for 24 h and analyzed as described by Kuang et al. (6).

RNA analysis. RNA was harvested from lung cells and processed using TRI reagent (Molecular Research Center, Cincinnati, OH) as described by Jean et al. (4). RNA (10 μg) was separated by agarose gel electrophoresis, transferred by capillary electrophoresis to a nylon membrane, and immobilized with UV light. The membrane was probed for expression of fibulin-5 and elastin mRNAs and 18S rRNA using probes labeled with [^{32}P]dCTP.

Protein analysis. Total protein was extracted from lung or lung cells with a detergent buffer consisting of 150 mM NaCl, 0.5% deoxycholate, 1% Nonidet P-40, 0.1% SDS, and 50 mM Tris (pH 7.6), separated by SDS-polyacrylamide gel electrophoresis, and then electroblotted onto a nitrocellulose filter. The filter was probed for fibulin-5 protein using a 1:1,000 dilution of the affinity-purified antibody and detected with a 1:3,500 dilution of horseradish peroxidase-conjugated goat-anti-rabbit antibody. Signal was detected using the ECL kit according to the manufacturer's instructions (Amersham Pharmacia Biotech UK, Buckinghamshire, UK).

Cell surface elastin was analyzed by an elastase hydrolysis experiment. Confluent quiescent lung interstitial fibroblasts were washed twice with PBS before addition of 10 ml of elastase-PBS solution as either human neutrophil elastase (25 mg/ml) or PPE (25 mg/ml). After incubation at 37°C for 5–10 min, the cells were harvested on ice in detergent buffer and analyzed by Western blot.

Fibulin-5 expression in vivo was analyzed by immunohistochemistry. Lung was inflation-fixed with 4% paraformaldehyde, dehydrated in ethanol, and embedded in paraffin. Deparaffinized tissue sections were blocked with nonimmune rabbit sera, incubated with the polyclonal fibulin-5 antibody at dilutions of 1:100 and 1:200, rinsed, and stained for peroxidase activity with the Vectabond ABC kit. Unstained sections or sections stained with dilute hematoxylin were photographed in a Leitz Orthoplan microscope. Photographs were generated with the Improvision Open-Lab Users Software program (Quincy, MA).

Statistics. The data for the Northern blot from the elastase experiment were normalized to the 18S signal, and fold induction of elastin or fibulin-5 mRNA was determined relative to control, the normal lung at baseline. Fold induction over control was evaluated by a *t*-test, and a $P < 0.05$ was considered significant.

RESULTS

Fibulin-5 mRNA expression. To examine whether fibulin-5 was induced in a mouse model of elastase-induced emphysema, we isolated total RNA from these mice at 20 days after an intratracheal instillation of either saline or PPE and analyzed by Northern blot (Fig. 1). Fibulin-5 mRNA is induced 3.8 ± 0.4-fold over baseline ($P = 0.009$, $n = 3$) in the elastase-treated lung at *day 20*. Elastin mRNA is elevated only 2.4 ± 0.5-fold ($P = 0.23$, $n = 3$) over baseline.

Fibulin-5 protein expression. Elastin is synthesized primarily by lung interstitial fibroblasts. To examine whether these fibroblasts synthesize fibulin-5 protein, we prepared a cellular lysate from normal neonatal rat lung and from quiescent confluent rat lung interstitial fibroblasts and analyzed fibulin-5 expression by Western blot as described in METHODS (Fig. 2). Lung interstitial fibroblasts contain lipid droplets that permit selective isolation from other lung mesenchymal cells. A prominent signal was detectable in a whole lung lysate, and this was enriched in a lung interstitial fibroblast lysate.

Fibulin-5 is a secreted protein and is reported to accumulate in the medium of cells in vitro (11). To examine whether fibulin-5 accumulates in the culture media of lung interstitial fibroblasts, a serum-reduced (0.4%) medium was harvested from confluent quiescent cells, concentrated, and analyzed by Western blot (Fig. 3). No signal was detected for the mature 67-kDa form of fibulin-5, but a prominent signal was present at ~21 kDa. The intensity of this signal increased as larger volumes of media were analyzed and decreased following treatment of the fibroblasts with IL-1β.

To examine whether mature fibulin-5 remained cell associated, we performed an elastase hydrolysis experiment (Fig. 4). A 67-kDa signal for mature fibulin-5 protein was detected in the cell layers that were washed solely with PBS. This signal was absent from those cells treated briefly with elastase (5 min). A nonspecific cross-reacting signal was also present and

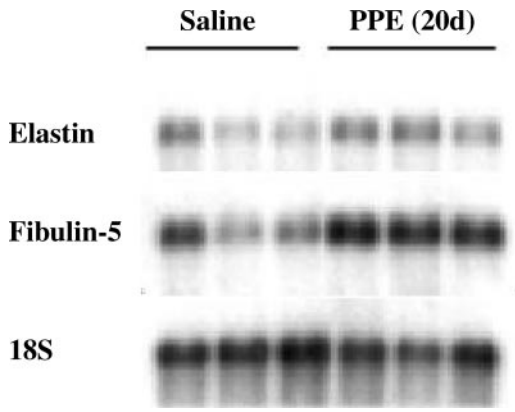

Fig. 1. Induction of fibulin-5 mRNA. C57/b mice were treated with saline or elastase [porcine pancreatic elastase (PPE)]. RNA was harvested from the lungs 20 days (d) following elastase treatment and analyzed as described in METHODS. This blot was probed for elastin and fibulin-5 mRNA expression. A probe for 18S rRNA was used to assess loading.

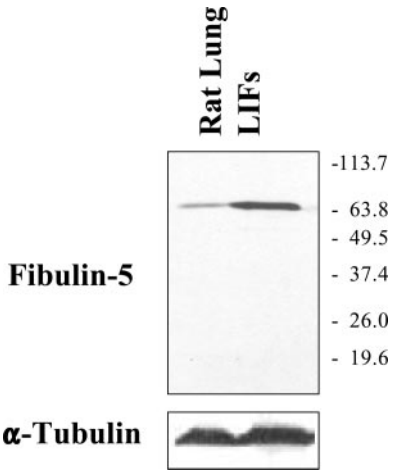

Fig. 2. Fibulin-5 protein expression in fibroblasts. A protein extract was prepared from isolated rat lung interstitial fibroblasts (LIF) and analyzed for fibulin-5 expression by Western blot as described in METHODS. Protein loading and integrity was assessed by expression of α-tubulin.

indicated that not all protein was digested by the elastase.

To determine the cellular site of fibulin-5 expression following injury in vivo, we compared normal and injured mouse lung by immunohistochemistry after fixation with paraformaldehyde (Fig. 5). The adult rat lung was examined as a positive control for fibulin-5 protein expression, as we have previously shown the presence of fibulin-5 mRNA expression in the pulmonary vasculature by in situ hybridization (4). No signal is evident when primary antibody is omitted as a negative control (Fig. 5*A*). A fibulin-5 signal is evident only in the blood vessel wall in the

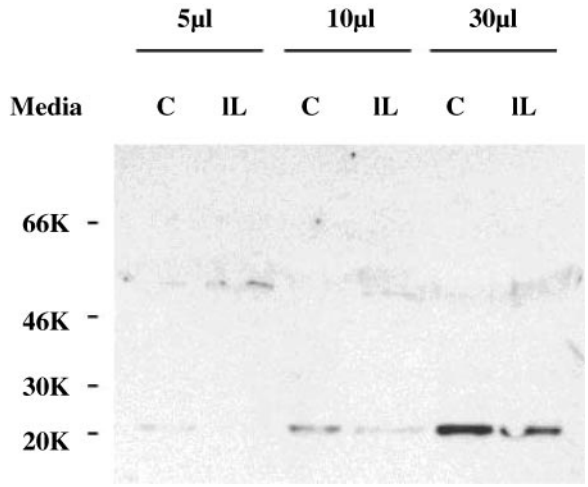

Fig. 3. Absence of fibulin-5 protein in culture media from fibroblasts. The media from rat interstitial cells in culture were harvested and concentrated as described in METHODS. Increasing aliquots were assessed for fibulin-5 protein expression by Western blot. Protein standards are labeled at *left*. No signal is evident at 67 kDa, the expected mass of fibulin-5, but a signal is present at ~21 kDa. This signal becomes more intense as increasing volumes of media are added, and this intensity is reduced by treatment with IL-1β (IL). C, control.

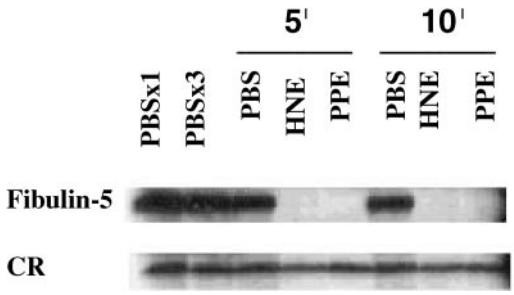

Fig. 4. Fibulin-5 remains associated with the fibroblast cell surface. Cell layers of rat interstitial fibroblasts were treated with PBS or human neutrophil elastase (HNE) or PPE. Protein was extracted and assayed for fibulin-5 expression by Western blot as described in METHODS. A cross-reacting signal (CR) demonstrates presence of an elastase-resistant band that serves as a loading control.

rat lung positive control (Fig. 5, *B* and *C*) and in the control mouse lung (Fig. 5*D*). Air space enlargement is evident in the lung at 20 days after elastase treatment (Fig. 5*E*). A fibulin-5 signal persists in the blood vessel wall (data not shown) but is now also present in the alveolar wall (Fig. 5*F*).

Regulation of fibulin-5 mRNA expression. IL-1β decreases elastin mRNA expression by inhibiting its transcription in lung interstitial fibroblasts (6). To examine whether IL-1β treatment affects fibulin-5 mRNA expression, we treated quiescent fibroblasts with IL-1β or IFN-γ, and RNA was harvested and analyzed (Fig. 6). Treatment with IL-1β alone and with IL-1β in combination with IFN-γ completely abolished fibulin-5 mRNA expression. IFN-γ alone did not affect fibulin-5 or elastin (not shown) mRNA expression.

DISCUSSION

Fibulin-5 gene expression is critical to elastogenesis as revealed by the elastinopathy observed in the skin, the vasculature, and the lung in fibulin-5 null mice. The ability of fibulin-5 to bind both cell surface integrins and elastin appears to be essential for extracellular elastic fiber organization (11, 19). Elastin expression in the lung is required during normal lung development and following experimental lung injury, for its impairment leads to air space enlargement resembling emphysema (reviewed in Refs. 3 and 15). Activation of connective tissue matrix production may serve a key role to limit air space enlargement after proteolytic lung injury. Inhibition of this response by inflammation may worsen emphysematous alterations in lung structures. Resistance of combined IL-1β and TNF-α receptor-deficient mice to elastase-induced emphysema strongly supports this hypothesis.

Myofibroblasts isolated from the postnatal lung during rapid alveolarization produce high levels of elastin (1). We now find that these cells also express fibulin-5 mRNA and protein in vitro. Interstitial fibroblasts in the normal adult lung do not express this mRNA determined by in situ hybridization. But expression can be reactivated in an injury model of emphysema. The level of induction is likely modulated locally by cytokines as exposure of interstitial fibroblasts to IL-1β

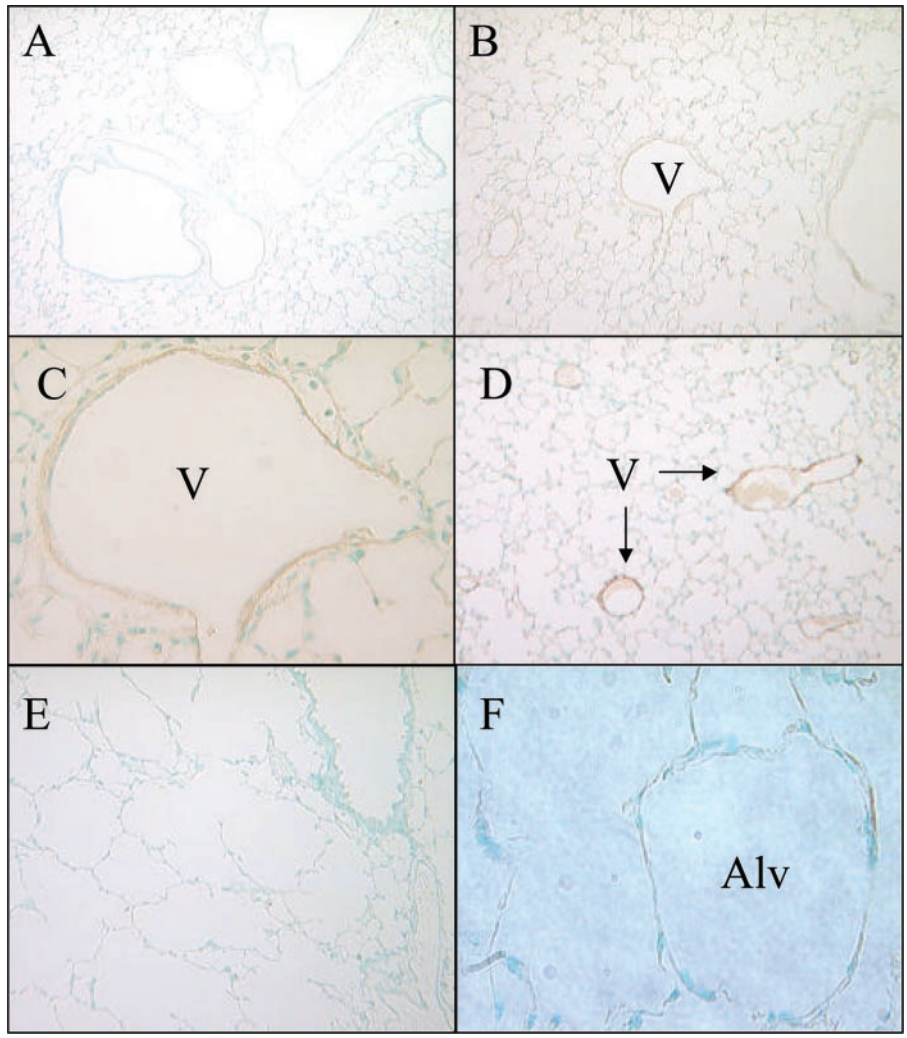

Fig. 5. Fibulin-5 protein induction following elastase-induced lung injury. Normal and elastase-treated mouse lung (*day 20* after injury) was prepared for immunohistochemistry as described in METHODS. The primary antibody was omitted in *A* as a negative control (×10). Normal rat lung is shown in *B* (×10) and *C* (×40), where the color brown denotes fibulin-5 signal limited to the vessel wall. Control mouse lung is shown in *D* (×10). Elastase-treated lung is shown in *E* (×10) and *F* (×100). Air space enlargement is evident, and brown signal is present in the alveolar (alv) wall. V, blood vessel.

dramatically downregulates fibulin-5 mRNA abundance. Elastin mRNA was also decreased by IL-1β exposure, although to a lesser degree. Formation of the normal elastin network in alveolar structures requires coordinated production of both elastin and fibulin-5 by interstitial fibroblasts. Together, our data indicate that proteolytic injury activates synthesis of both of these matrix components in vivo and that the associated inflammatory process further regulates their expression.

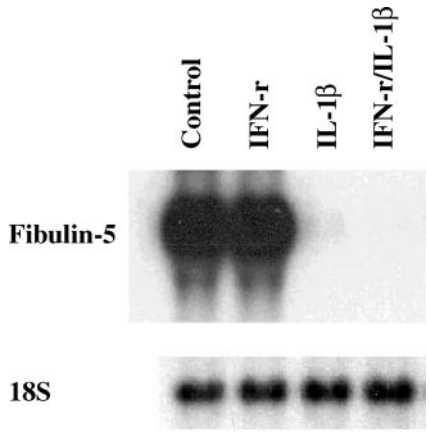

Fig. 6. Regulation of fibulin-5 mRNA by IL-1β. Rat lung interstitial fibroblasts were harvested, placed in culture, and treated with saline or cytokines as described in METHODS. RNA was isolated and analyzed for fibulin-5 mRNA expression. A probe for 18S rRNA was used to assess loading.

The matrix-related genes for elastin and fibulin-5 are both developmentally regulated in the late fetal lung (4, 14). Elastin, a major structural protein of the lung, is found in the alveolar wall, derived from interstitial fibroblasts, as well as in the blood vessels, as a product of smooth muscle and endothelial cells (14). We originally identified fibulin-5 (DANCE) as a novel developmentally regulated gene in the newborn lung using suppression subtractive hybridization. The tropoelastin gene was also included among our original clones from this screen. In the developing and growing lung, fibulin-5 expression predominated in endothelial cells, as assessed by in situ hybridization. This suggested a major role for fibulin-5 in lung vascular development. Fibulin-5 was also evident in the interstitium. Our new data clearly indicate that primary lung fibroblasts express fibulin-5 mRNA and confirm our initial observation.

We have also shown that lung interstitial fibroblasts express fibulin-5 protein. The protein's abundance is enriched in the primary cell isolates over that of the whole lung. Fibulin-5 was initially characterized as a secreted protein (DANCE), and it accumulated in the medium when overexpressed in 293 and COS7 cells. This protein was also shown to mediate endothelial cell attachment in vitro via its RGD domain (12). However, fibulin-5 protein did not accumulate in the medium of our lung interstitial fibroblast cultures. Rather, it remained associated with the fibroblast cell surface. Fibulin-5 is known to bind the integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_9\beta_1$ (11), and human fibroblasts are known to express $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (16). Our data suggest that rat lung interstitial fibroblasts contain sufficient cell surface-binding sites, likely integrins, so that all available fibulin-5 is bound and remains cell associated. However, we do not know if the binding between fibulin-5 and elastin represents an intracellular event within the secretory pathway or an extracellular one whereby fibulin-5 recruits soluble tropoelastin to the cell surface. Further studies will be required to address this. Nor do we yet know the nature of the 21-kDa band that we detected in the media from the fibroblast cultures. Although this signal may represent a cross-reacting protein, no such cross-reacting band was observed in the medium of COS or Chinese hamster ovary cells overexpressing fibulin-5 (12). Alternatively, this band could represent a unique protein fragment derived from fibulin-5 expression in these fibroblasts. In support of this hypothesis, our present data show that the intensity of the fragment, as well as that of fibulin-5 mRNA, decreased following treatment of our fibroblast cultures with IL-1β.

Characterization of factors that regulate fibroblast elastin gene expression is a major focus of this laboratory. Treatment of lung fibroblasts with insulin-like growth factor (13), transforming growth factor-β (TGF-β) (10), and retinoic acid (7) can induce elastin mRNA expression. In contrast, basic fibroblast growth factor (2) and IL-1β downregulate its expression (6). The inhibitory effect of IL-1β occurs at the level of elastin gene transcription. The mechanism involves the activation and translocation of the p65 subunit of NF-κB into the nucleus and its interaction with Sp1, which binds to a *cis*-acting element located between nucleotides −118 and −102 in the elastin gene promoter (6). Overall, this IL-1β response could potentiate inflammation and compromise repair in the injured alveolar wall. To explore this further, we examined the effect of IL-1β on fibulin-5 mRNA expression in lung interstitial fibroblasts. Indeed, we found that IL-1β dramatically decreased the fibulin-5 mRNA signal. Further studies are now in progress to determine whether the fibulin-5 promoter is regulated in the same fashion similar as we described for elastin.

Recent studies have also shown that TGF-β can induce fibulin-5 gene expression in 3T3-L1 fibroblasts (17). Basal expression of fibulin-5 mRNA is undetectable in these cells, as we also observed in IMR90 fibroblasts (4). TGF-β induced fibulin-5 mRNA in a dose- and time-dependent fashion. Fibulin-5, in turn, induced a proliferative response via activation of ERK1/2 and p38 mitogen-activated protein kinase pathways. In fibrosarcoma cells, fibulin-5 also increased fibronectin-directed migration and invasiveness through synthetic basement membranes. In contrast, fibulin-5 mRNA was downregulated in several metastatic cancers and induced an antiproliferative response when expressed in mink lung Mv1Lu epithelial cells. The authors proposed that fibulin-5 expression could have context-specific effects on cell proliferation, motility, and invasion (17).

In summary, our studies have found coordinate expression of fibulin-5 and elastin in lung fibroblasts. Examination of the fibulin-5 promoter will ultimately provide new clues into the signaling pathways that regulate fibulin-5 gene expression. Such studies should provide new insight into the role of elastogenesis during lung alveolar development and alveolar repair after injury. Agents that mimic the effects of fibulin-5 may even provide new tools to manipulate the outcome of lung injury in favor of normal repair rather than emphysema.

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DISCLOSURES

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