A β1 integrin phosphatidylinositol 3-kinase/Akt pathway regulates fibroblast survival in collagen matrices. When fibroblasts attach to collagen, Akt becomes phosphorylated, providing a survival signal. In contrast, in response to mechanical forces generated during collagen contraction, Akt is dephosphorylated and fibroblasts undergo apoptosis. The kinase(s) responsible for regulating Akt phosphorylation in response to matrix-derived mechanical signals are unclear. Integrin-linked kinase (ILK) is associated with the β1 integrin in the focal adhesion complex and as such is a candidate kinase that may regulate Akt phosphorylation and fibroblast viability. Nevertheless, there is no direct evidence that matrix-derived mechanical forces regulate cell viability by modulating ILK activity. Here, we show that ILK activity decreased in response to collagen matrix contraction, which correlated with Akt dephosphorylation and induction of fibroblast apoptosis. In contrast, enforced activation of β1 integrin by activating antibody preserved ILK and Akt activity during collagen matrix contraction, and this is associated with protection from collagen contraction-induced apoptosis. Knock-down of ILK by small, interfering RNA (siRNA) attenuated Akt phosphorylation in response to ligation of β1 integrin by collagen or activating antibody and enhanced fibroblast apoptosis in response to collagen contraction. Kinase dead ILK attenuated Akt phosphorylation and enhanced fibroblast apoptosis, whereas hyperactive and wild type ILK augmented Akt phosphorylation and protected fibroblasts from apoptosis. Constitutively active Akt preserved Akt activity and rescued ILK siRNA-treated fibroblasts from collagen contraction-induced apoptosis. These data establish that matrix-derived mechanical forces sensed by β1 integrin are capable of modulating ILK activity which regulates fibroblast viability via an Akt-dependent mechanism.

During tissue development and repair, cells respond to cues derived from the extracellular matrix (ECM), and in the process such cellular functions as cell viability may be modulated.

As an example, in the early phase of tissue repair, proliferating fibroblasts deposit type I collagen in the wound space. However, during resolution of tissue repair fibroblasts contract the type I collagen matrix and subsequently undergo apoptosis (1). Although the precise mechanism governing the removal of fibroblasts during tissue repair is unclear, because fibroblasts are removed upon wound contraction biophysical signals derived from the ECM have been implicated as an important determinant (2, 3). The ability of the cell to detect alterations in the ECM and respond accordingly is mediated through integrins. Integrins are cell surface receptors that link the ECM with intracellular signaling molecules and the actin cytoskeleton (4, 5). ECM-integrin interaction may influence cell viability via at least two basic mechanisms. First, direct ligation of integrin by the ECM promotes anchorage-dependent cell survival (4, 5). Second, integrins also function as mechanoreceptors, and as such are capable of detecting matrix-derived mechanical signals that regulate cell viability (3, 6–11).

Mirroring the physiologic elimination of fibroblasts upon wound contraction, fibroblasts in three-dimensional collagen gels undergo apoptosis in response to collagen matrix contraction (1, 2, 3, 11, 12). We have employed the three-dimensional collagen gel model to study the molecular mechanism by which collagen matrix contraction regulates fibroblast viability. We have found that in response to mechanical forces generated during collagen matrix contraction, a β1 integrin/PI 3-kinase/Akt signal pathway regulates fibroblast viability (3, 11). During collagen matrix contraction FAK and Akt become dephosphorylated, and fibroblasts subsequently undergo apoptosis (11). Modulation in FAK and Akt activity are mediated through the β1 integrin, as enforced activation of β1 integrin by activating β1 integrin antibody up-regulates FAK and Akt activity and protects fibroblasts from contraction-induced apoptosis (11). Within the type I collagen matrix, FAK functions as an integrin-associated signaling molecule that is upstream of PI 3-kinase/Akt. FAK transduces type I collagen-β1 integrin viability signals that modulate PI 3-kinase and Akt activity.

The identities of β1 integrin-associated signaling molecule(s) downstream of PI 3-kinase that are responsible for directly modulating Akt phosphorylation in response to collagen contraction-derived mechanical signals are not known. It has been demonstrated that ligation of β1 integrin by type I collagen or enforced activation of β1 integrin by β1 integrin-activating antibody phosphorlylates serine 473 of Akt corresponding to full activation of the kinase (3). However, the precise kinase(s) responsible for phosphorylation of serine 473 of Akt remain unclear. One such candidate kinase that has been implicated in direct phosphorylation of serine 473 of Akt is integrin-linked kinase or ILK (13–16). ILK is a serine-threonine kinase that couples integrins to downstream signaling pathways that regulate a variety of cellu-
lar functions including cell viability. In this respect, in some systems ILK has been shown to function downstream of PI 3-kinase in promoting phosphorylation of serine 473 of Akt (14–16). The mechanism by which ILK may facilitate Akt phosphorylation is controversial. Several studies suggest that ILK activity may be responsible for phosphorylating serine 473 of Akt (13–16). However, other studies indicate that ILK functioning as an adapter protein rather than a kinase may facilitate activation of Akt (17, 18). Moreover, a recent report found that ILK was capable of regulating endothelial cell survival in an Akt-independent manner (19). Thus, the role of ILK in regulating cell survival appears to be complex and, depending on the cell system being analyzed, may involve Akt-dependent and -independent mechanisms. Multiple exogenous stimuli have been shown to activate ILK; these include direct integrin ligation by the ECM and growth factor and chemokine activation (20, 21). Although these data suggest a possible link between ILK activity and the regulation of Akt phosphorylation, currently there is no direct evidence that matrix-derived mechanical forces sensed by integrin receptors modulate ILK activity and cell viability in an Akt-dependent manner.

In the present study, we present data establishing that collagen matrix contraction—derived mechanical signals sensed by β1 integrin are capable of modulating ILK activity, which regulates fibroblast viability through an Akt-dependent mechanism.

MATERIALS AND METHODS

Cell Culture—Human lung fibroblasts (CCL-210, American Type Culture Collection, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium (Sigma) containing 10% heat-inactivated fetal calf serum and used between passages 9 and 11.

Antibodies and Reagents—TS2/16 β1 integrin-activating antibody was produced from hybridoma cell culture (ATCC, HB-243). Mouse monoclonal antibody P2D2 (raised against the human β1 integrin subunit) was provided by Dr. Leo Furcht (University of Minnesota). Anti-polyclonal Akt antibody, anti-phosphorylated Akt antibody, the cleaved form of caspase-9 antibody, GSK3β antibody, and ILK antibody were purchased from Cell Signaling (Beverly, MA). Anti-monoconal ILK for immunoprecipitation was from Upstate Biotechnology (Lake Placid, NY). Wortmannin was from Sigma-Aldrich, and anti-monoclonal β1 integrin antibody was purchased from Chemicon (Temecula, CA). Myc-His-tagged Akt1 (activated), hyperactive ILK cDNA (S343D mutation), and kinase dead ILK (E359K) in pUSE amp vector under the control of 1.5× ThermoTag promoter were purchased from Upstate Biotechnology.

ILK and Control siRNA—siRNA duplexes were synthesized by Qiagen-Xeragon (Germantown, MD). Twenty-one base sequences of the human ILK gene targeting the pleckstrin homology domain of ILK (ILK-H) were chosen as described previously with minor modifications (22). ILK sense siRNA sequence is 5’ GGU CAA CGG AGA UCA CUC GUU GAG CdTT 3’; ILK antisense siRNA sequence is 5’ AGA GUG AUU CUC GUU GAG CdTT 3’. A control non-silencing siRNA was derived from the manufacturer’s instructions.

Immunoprecipitation and Western Analysis—Fibroblasts were lysed in lysis buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 0.5% sodium deoxycholate, 1% Nonidet P-40, 2.5 mM Na3P2O7, 1 mM glycerolphosphate, 1 mM Na3VO4, 1× protease inhibitor mixture (Calbiochem), and 1 mM phenylmethylsulfonyl fluoride. Lysates were precleared for 1 h at 4 °C with protein G-coupled agarose beads and immunoprecipitated overnight (16 h) at 4 °C with the appropriate primary antibody. Western analysis of human lung fibroblasts was performed as described previously (3). Briefly, equal amounts of protein from cell lysates were subjected to 8–10% SDS-PAGE and transferred (20 min, 24 V) to nitrocellulose membrane. Membranes were blocked with 20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.05% Tween 20 containing 6% nonfat dry milk, incubated with the primary antibody, washed, and incubated with horseradish peroxidase-conjugated secondary antibody. The membranes were developed using the ECL method (Amersham Biosciences).

In Vitro ILK Assay—Human lung fibroblasts were transfected with ILK or control siRNA. Alternatively, cells were transfected with hyperactive ILK or kinase dead ILK. Cells were lysed and immunoprecipitated with 4 μg of anti-ILK antibody. 1 μg of GSK3β fusion protein (Cell Signaling) was then added to the immunoprecipitated lysates in 1× kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerolphosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, 10 mM MgCl2) followed by incubation for 30 min at 30 °C. After reaction, 20 μl of 2× SDS sample buffer (187.5 mM Tris-HCl, pH 6.8, 6% SDS, 30% glycerol, 150 mM dithiothreitol, 0.03% bromophenol blue) was added to terminate reaction. Samples were analyzed by Western analysis with phospho-GSK3β and total GSK3β antibodies.

Anoikis Assay—Anoikis assays were performed as described by Frisch and Francis (23). Tissue culture plates were coated twice with poly-DMEM (4 mg/ml each; Sigma) and seeded with cells grown in poly-DMEM for 18–24 h. Cells were then treated with wounding solution (22, 25). Within the first hour of fibroblast incorporation into collagen gels, both ILK and Akt activity increased slightly (Fig. 1). This correlated with initial fibroblast attachment and spreading within the matrix. However, within 3 h, ILK activity decreased, which correlates with dephosphorylation of Akt and induction of apoptosis (3). To begin to assess whether changes in ILK activity modulate Akt activity and fibroblast viability, we first examined ILK activity as a function of time during collagen matrix contraction. Fibroblasts incorporated into three-dimensional collagen gels were lysed directly in the gels at various times during contraction. ILK was immunoprecipitated, and phosphorylation of GSK3β and total GSK3β were examined by Western analysis. ILK phosphorylates serine 9 of GSK3β and therefore has been used as a surrogate marker of ILK activity (22, 25). Within the first hour of fibroblast incorporation into collagen gels, both ILK and Akt activity increased slightly (Fig. 1A). This correlated with initial fibroblast attachment and spreading within the matrix. However, within 3 h, ILK activity had begun to decline; this decline correlated with the onset of collagen matrix contraction (Fig. 1B). Akt activity was still preserved at the 3-h time point. Moreover, as collagen matrix contraction progressed, ILK activity became markedly suppressed, and this was associated with a progressive decline in Akt activity. Note that by 22 h, a time point at which we had previously found that collagen matrix contraction is mostly
completed and fibroblast apoptosis is triggered (3, 11), both ILK and Akt activity were largely abrogated. In contrast, ILK and Akt activity increased in non-contractile collagen gels as a function of time (Fig. 1C). This is consistent with our previous finding that Akt activity does not decrease under non-contractile conditions and that fibroblasts remain viable (3, 11). These data indicate that collagen contraction down-regulates ILK activity. The correlation between the decline in ILK and Akt activity, with decreases in ILK activity preceding Akt, was consistent with a causal role for ILK in regulating fibroblast viability in contractile collagen matrices via modulation of Akt activity.

Ligation of β1 Integrin by Type I Collagen or by β1 Integrin-activating Antibody Phosphorylates Serine 473 of Akt in a PI 3-Kinase-dependent Fashion—We have previously shown that fibroblast viability in type I collagen matrices is regulated by the β1 integrin and involves a PI 3-kinase/Akt signaling pathway (3). Upon ligation of β1 integrin by type I collagen or by β1 integrin-activating antibody, serine 473 of Akt becomes phosphorylated in a PI 3-kinase-dependent fashion indicating full activation of the kinase (3, 11) (Fig. 2). Therefore, we sought to examine the effect of ligation of β1 integrin by collagen or by activating antibody on Akt activity. Serum-starved fibroblasts were plated on type I collagen dishes, and phosphorylation of serine 9 of GSK3β and total GSK3β levels were examined. We found that phosphorylation of serine 9 of GSK3β increased as a function of time as fibroblasts spread and adhered on type I collagen (Fig. 3A, left panel). Total GSK3β levels did not change. The increase in phosphorylation of serine 9 of GSK3β could be inhibited by blocking β1 integrin-type I collagen interaction by preincubating the cells with β1 integrin-blocking antibody (Fig. 3A, right panel). These data indicate that ILK activity increases as fibroblasts adhere to type I collagen and that the increase in ILK activity is dependent on β1 integrin-type I collagen interaction. As shown in Fig. 2, enforced activation of β1 integrin by β1 integrin-activating antibody promotes phosphorylation of serine 473 of Akt (3). Therefore, we sought to examine the effect of ligation of β1 integrin by activating antibody on Akt activity. In these experiments serum-starved fibroblasts were plated in suspension culture, β1 integrin-activating antibody was added to the cells in suspension, and GSK3β phosphorylation was measured. Similar to ligation of β1 integrin with type I collagen, we found that enforced activation of β1 integrin by β1 integrin-activating antibody increased phosphorylation of serine 9 of GSK3β (Fig. 3B, left panel). To determine whether this increase in Akt activity was dependent upon PI 3-kinase, the cells were preincubated with wortmannin prior to ligation of β1 integrin by β1 integrin-activating antibody. Wortmannin effectively inhibited the increase in Akt activity in response to enforced activation of β1 integrin-activating antibody (Fig. 3B, right panel). These data suggested that ILK may be downstream of PI 3-kinase in mediating phosphorylation of serine
human lung fibroblasts were serum-starved for 48 h and plated on type I collagen-coated dishes (100 µg/ml) for 30 min. Cells were collected, lysed and immunoprecipitated (IP) with anti-ILK antibody (4 µg/ml), and probed with phosphorylated GSK3β (Ser-9) and total GSK3β antibodies as indicated. As a positive control, serum-starved cells (S) were treated with 10% serum for 30 min, and ILK activity was assessed. Right panel, cells were preincubated with β1 integrin-blocking antibody (10 µg/ml) for 30 min and plated on type I collagen-coated dishes (100 µg/ml) for the indicated times. Cells were then lysed, and the lysates immunoprecipitated with anti-ILK antibody (4 µg/ml) and probed with phosphorylated GSK3β (Ser-9) and total GSK3β antibodies as indicated. WB, Western blot. B, normal human lung fibroblasts were serum-starved for 48 h and placed in suspension culture. Left panel, the cells in suspension culture were treated with β1 integrin-activating antibody for the indicated times and immunoprecipitated with anti-ILK antibody (4 µg/ml), and the levels of phosphorylated GSK3β (Ser-9) or total GSK3β were determined by Western analysis. Right panel, the cells were preincubated in the presence of wortmannin (200 nM) for 30 min. The cells were then treated with 10 µg/ml β1 integrin-activating antibody, immunoprecipitated with anti-ILK antibody (4 µg/ml), and probed with phosphorylated GSK3β (Ser-9) and total GSK3β antibodies as indicated.

FIG. 3. Ligation of β1 integrin by type I collagen or β1 integrin-activating antibody increases ILK activity. A, left panel, normal human lung fibroblasts were serum-starved for 48 h and plated on type I collagen-coated dishes (100 µg/ml) for 30 min. Cells were collected, lysed and immunoprecipitated (IP) with β1 integrin-activating antibody (10 µg/ml), and probed with antibody against phosphorylated GSK3β (Ser-9) and total GSK3β antibodies as indicated. As a positive control, serum-starved cells (S) were treated with 10% serum for 30 min, and ILK activity was assessed. Right panel, cells were preincubated with β1 integrin-blocking antibody (10 µg/ml) for 30 min and plated on type I collagen-coated dishes (100 µg/ml) for the indicated times. Cells were then lysed, and the lysates immunoprecipitated with anti-ILK antibody (4 µg/ml) and probed with phosphorylated GSK3β (Ser-9) and total GSK3β antibodies as indicated. WB, Western blot. B, normal human lung fibroblasts were serum-starved for 48 h and placed in suspension culture. Left panel, the cells in suspension culture were treated with β1 integrin-activating antibody for the indicated times and immunoprecipitated with anti-ILK antibody (4 µg/ml), and the levels of phosphorylated GSK3β (Ser-9) or total GSK3β were determined by Western analysis. Right panel, the cells were preincubated in the presence of wortmannin (200 nM) for 30 min. The cells were then treated with 10 µg/ml β1 integrin-activating antibody, immunoprecipitated with anti-ILK antibody (4 µg/ml), and probed with phosphorylated GSK3β (Ser-9) and total GSK3β antibodies as indicated.

473 of Akt in response to ligation of β1 integrin by type I collagen or enforced activation of β1 integrin by β1 integrin-activating antibody.

Ligation of β1 Integrin with β1 Integrin-activating Antibody Promotes Association of ILK with β1 Integrin and Akt—Following ligation of β1 integrin, ILK can be found in the focal adhesion complex associated with β1 integrin. Therefore, we sought to determine whether enforced activation of β1 integrin by β1 integrin-activating antibody would promote the association of ILK with β1 integrin. Serum-starved fibroblasts were treated with β1 integrin-activating antibody, ILK was immunoprecipitated, and β1 integrin was immunoblotted. The physical association of ILK with β1 integrin increased in a time-dependent manner (Fig. 4A). These data demonstrate that ILK is recruited to β1 integrin in response to ligation with β1 integrin-activating antibody. We next investigated the effect of ligation of β1 integrin by activating antibody on the association of ILK with Akt. The association of ILK with Akt increased in a time-dependent fashion, and this interaction correlated with the degree of phosphorylation of Akt (Fig. 4B). These data indicate that ligation of β1 integrin by activating antibody promotes the interaction of ILK with β1 integrin and the association of ILK with Akt, and this correlates with phosphorylation of Akt. Therefore, these results, combined with our previous data demonstrating that enforced activation of β1 integrin by β1 integrin-activating antibody promotes both an increase in ILK activity and the phosphorylation of serine 473 of Akt, suggested a link between the increase in ILK activity and phosphorylation of Akt.

Enforced Activation of β1 Integrin by β1 Integrin-activating Antibody Inhibits the Decrease in ILK and Akt Activity Associated with Collagen Matrix Contraction—We have previously shown that enforced activation of β1 integrin by addition of activating antibody to the collagen gels preserves Akt activity and protects fibroblasts from collagen matrix contraction-induced apoptosis (3). Because ligation of β1 integrin by activating antibody also increases ILK activity, we sought to examine the effect of activating antibody on ILK and Akt activity during collagen matrix contraction. Lung fibroblasts were incorporated into collagen matrices. Prior to gel contraction (1 h after incorporation of fibroblast into the matrices), β1 integrin-activating antibody was added to the collagen gels. ILK and Akt activity was determined as a function of time during collagen contraction. In contrast to collagen gels not treated with activating antibody, where ILK and Akt activity are suppressed...
during contraction (see Fig. 1), enforced activation of β1 integrin by activating antibody inhibited the suppression in ILK and Akt activity associated with collagen matrix contraction (Fig. 5). Control IgG antibody had no effect on ILK and Akt activity or fibroblast viability in contractile collagen gels (data not shown). These data, combined with our previously published results, suggest that collagen contraction-related mechanical signals down-regulate a β1 integrin survival signal pathway by decreasing ILK and Akt activity. Enforced activation of β1 integrin by activating antibody preserves this signal pathway and protects fibroblasts from apoptosis.

**ILK siRNA Knocks Down ILK mRNA, Protein Expression, and Akt Activity and Inhibits the Increase in Akt Activity Brought about by Ligation of Type I Collagen and β1 Integrin-activating Antibody**—To directly assess the role of ILK in regulating phosphorylation of serine 473 of Akt in response to ligation of β1 integrin by type I collagen or enforced activation of β1 integrin by β1 integrin antibody, we examined the effect of down-regulation of ILK by double-stranded RNA interference (siRNA) on Akt phosphorylation. To knock down ILK expression we used an ILK siRNA, which targets the pleckstrin homology domain of ILK and has been previously shown effective in silencing ILK (22). A non-silencing siRNA was used as a control. To confirm that the ILK siRNA knocks down ILK expression, we examined ILK mRNA levels by reverse transcriptase-PCR analysis and ILK protein levels by Western analysis. To perform these preliminary experiments, we used non-serum-starved fibroblasts in order to optimize ILK expression and facilitate analysis of the effectiveness of ILK siRNA on knocking down ILK expression and activity. Lung fibroblasts treated with ILK or control siRNA were grown on tissue culture plates. Both ILK mRNA levels and ILK protein levels were knocked down in response to 100 nM ILK siRNA but not with the non-silencing control (Fig. 6, A and B). The effect of ILK siRNA on ILK activity was examined by measuring the level of phosphorylation of a GSK3β fusion protein using an *in vitro* kinase assay (14, 26). ILK siRNA decreased ILK activity but not control (Fig. 6C). Furthermore, using Lipofectamine we examined the effect of transfection of ILK siRNA into lung fibroblasts on the level of Akt phosphorylation. ILK siRNA suppressed phosphorylation of serine 473 of Akt, whereas the non-silencing control had no effect on the level of Akt phosphorylation (Fig. 6B). These data demonstrate that ILK siRNA effectively knocks down ILK protein expression and activity and inhibits Akt phosphorylation.

Having confirmed that ILK siRNA functionally knocks down ILK, we next wanted to examine the effect of the knock-down of ILK activity on Akt phosphorylation in response to ligation of β1 integrin by type I collagen or enforced activation of β1 integrin by β1 integrin antibody. To perform these experiments, ILK or control siRNA-transfected human lung fibroblasts were serum-starved for 48 h. Cells were then plated on type I collagen-coated dishes for 30 min. ILK siRNA knocked down ILK protein expression and inhibited the increase in Akt phosphorylation in response to ligation of β1 integrin by type I collagen. Control siRNA did not affect the increased level of ILK protein expression or Akt phosphorylation associated with plating the cells on collagen (Fig. 6D). We also investigated the effect of ILK siRNA on Akt activity in response to ligation of β1 integrin by activating antibody. ILK siRNA effectively knocked down the increase in Akt phosphorylation in response to the activating antibody. Control siRNA had no effect on Akt phosphorylation (Fig. 6E). As a control, we examined the level of phospho-Akt, total Akt, and total ILK under basal conditions (basal integrin activation). Basal conditions consisted of ILK or control siRNA-treated fibroblasts that were serum-starved and plated on uncoated tissue culture plates (Fig. 6D). ILK siRNA also decreased the basal level of Akt phosphorylation associated with attachment to tissue culture plate. These data demonstrate that ILK plays a role in regulating Akt activity in response to ligation of β1 integrin by type I collagen or β1 integrin-activating antibody.

**Knock-down of ILK Decreases Akt Phosphorylation and Augments the Level of Apoptosis of Fibroblasts in Type I Collagen Matrices**—We have previously shown that fibroblasts undergo apoptosis in response to type I collagen matrix contraction. In response to collagen contraction, Akt becomes dephosphorylated. However, up-regulation of Akt activity by enforced activation of β1 integrin by activating antibody or by ectopic expression of constitutively active PI 3-kinase protects fibroblasts from apoptosis (3, 11). Therefore, we examined the effect of knock-down of ILK on fibroblast viability in type I collagen matrices. We have previously shown that in response to collagen matrix contraction, Akt becomes dephosphorylated and fibroblasts spontaneously undergo apoptosis. Human lung fibroblasts were transfected with ILK or control siRNA and incorporated into contractile type I collagen gels. The gels were allowed to contract for 48 h. The gels were then digested with collagenease, and the recovered cells were examined for the level of phosphorylated Akt and cleaved caspase-9. Previous studies have shown that ILK inhibition induces activation of caspase-9, promoting apoptosis (27, 28). Cell viability was assessed by TUNEL assay. We first examined the level of Akt phosphorylation following 48 h of collagen matrix contraction. The level of phosphorylated Akt was largely abrogated in fibroblasts transfected with ILK siRNA and subjected to collagen matrix contraction, compared with cells treated with control siRNA (Fig. 7A). We also measured the level of the cleaved form of caspase-9 in contractile collagen matrices gels in the presence or absence of ILK siRNA. The level of cleaved caspase-9 was increased in contractile gels treated with ILK siRNA compared with contractile gels treated with control siRNA. As a control, the levels of phosphorylated Akt and cleaved caspase-9 in untransfected fibroblasts recovered from collagen gels are shown (Fig. 7A). Fibroblast viability in contractile collagen matrices treated with ILK or control siRNA was evaluated by TUNEL assay at 48 h. Shown in Fig. 7B are fluorescent microscopic pictures of DAPI (left panel) and TUNEL (right panel) staining performed on fibroblasts recovered from collagen matrices. Note the increase in TUNEL positivity in cells transfected with ILK siRNA compared with control siRNA. Interestingly, we found that although the level of Akt phosphorylation was significantly decreased by ILK siRNA, the level of apoptotic fibro-
ILK Regulation of Cell Survival in Contractile Collagen Matrices

ILK Activity Regulates the Level of Akt Phosphorylation and Fibroblast Viability in Type I Collagen Matrices—Several studies suggest that ILK activity is required for phosphorylation of serine 473 of Akt (13, 14, 15, 22), whereas other studies using kinase dead ILK indicate that ILK functions as an adapter molecule, indirectly promoting Akt activation (17, 18). Our results suggest that ILK activity is responsible for regulating Akt phosphorylation and fibroblast viability. However, to address this issue directly, we examined the effect of kinase dead ILK and wild type or hyperactive ILK constructs and incorporated them into contractile collagen gels. At 48 h the cells were recovered and analyzed for ILK activity using the in vitro kinase assay. As shown in Fig. 8A at 48 h, in contrast to cells transfected with wild type ILK in which the level of ILK activity was slightly increased, no ILK activity could be detected in recovered cells transfected with kinase dead ILK. Furthermore, significantly elevated levels of phosphorylated serine 9 of GSK3β could be detected in the cells transfected with hyperactive ILK (Fig. 8B). The level of total GSK3β is shown as a loading control. Total Akt is shown as a loading control.

FIG. 6. ILK siRNA knocks down ILK mRNA, protein expression, and ILK activity and inhibits the increase in Akt activity brought about by ligation by type I collagen and β1 integrin-activating antibody. A, non-serum-starved human lung fibroblasts were transfected with 100 nM ILK or control siRNA. 24 h post-transfection, reverse transcriptase-PCR was performed to assess ILK mRNA expression. I, ILK siRNA; C, control siRNA; U, untransfected cells; N/C, negative control. B, non-serum-starved fibroblasts were transfected with ILK siRNA or control siRNA (0, 50, 100 nM). 24 h post-transfection, the cells were lysed and Western analysis was performed to assess ILK, phosphorylated Akt, (phospho-Akt-ser473), and total Akt levels. C, the effect of ILK siRNA on ILK activity was examined using an in vitro kinase assay. Fibroblasts were transfected with ILK siRNA or control siRNA (100 nM). 24 h post-transfection, the cells were lysed and immunoprecipitated with anti-ILK (phospho-Akt-ser473), and total Akt levels. D, western blot analysis from cells transfected with ILK siRNA or control siRNA showing total ILK and Actin as a loading control. E, human lung fibroblasts were transfected with ILK or control siRNA (100 nM) and then serum-starved for 48 h. The cells were plated on uncoated tissue culture plates (NT, control (not treated)) or type I collagen (100 μg/ml)-coated plates for 30 min. The cells were then lysed, and Western analysis was performed to assess the levels of phosphorylated Akt (phospho-Akt-ser473), total Akt, and total ILK. F, serum-starved fibroblasts transfected with ILK or control siRNA were treated with β1 integrin-activating antibody (10 μg/ml) for 30 min, and Western analysis was performed using the indicated antibodies.
TUNEL (right panels) staining of fibroblasts transfected with hyperactive ILK, kinase dead ILK, or untransfected cells recovered from contractile collagen matrices at 48 h. Note the paucity of TUNEL positive cells in fibroblasts treated with hyperactive ILK. In contrast, there was an increase in TUNEL positivity in fibroblasts transfected with the kinase dead construct. We found that after 48 h in contractile collagen gels, 35% of recovered fibroblasts transfected with kinase dead were apoptotic, whereas >10% of cells expressing hyperactive ILK were apoptotic. Approximately 18% of the untransfected cells recovered from collagen gel were apoptotic at 48 h (Fig. 8D). Collectively these data indicate that ILK functioning as a kinase regulates the level of Akt phosphorylation and fibroblast viability in collagen matrices.

Constitutively Active Akt Rescues ILK siRNA-treated Fibroblasts from Collagen Contraction-induced Apoptosis—Our data suggest that mechanical forces generated during collagen matrix contraction modulate ILK activity, which in turn regulates fibroblast viability by altering the level of Akt phosphorylation. However, a recent report found that ILK was capable of regulating endothelial cell survival in an Akt-independent manner (19). This leaves open the possibility that the ability of ILK to regulate fibroblast viability in our system may at least in part be due to an Akt-independent mechanism. To evaluate this possibility, the effect of constitutively active Akt on rescuing ILK siRNA-treated fibroblasts from the proapoptotic stimulus of collagen contraction was examined. Lung fibroblasts transfected with ILK or control siRNA and empty vector or constitutively active Akt were incorporated into contractile collagen gels. At 48 h after collagen matrix contraction, the level of phosphorylated Akt was examined. The level of phosphorylated Akt in cells transfected with constitutively active Akt and treated with ILK siRNA was relatively preserved compared with cells transfected with empty vector (Fig. 9A). The level of actin is shown as a loading control. Furthermore, at 48 h after contraction, the cells were recovered

**Fig. 7.** Knock-down of ILK decreases Akt phosphorylation and augments the level of apoptosis of fibroblasts in type I collagen matrices. A, human lung fibroblasts were transfected with either control siRNA or ILK siRNA (100 nM). 24 h post-transfection the cells were incorporated into collagen gels (0.5 mg/ml), and the gels were allowed to contract for 48 h. Shown is a Western blot demonstrating the levels of phosphorylated Akt (phospho-Akt-ser473) and cleaved caspase-9 in control siRNA and ILK siRNA-transfected fibroblasts at 48 h in contractile collagen gels. Actin is shown as a loading control. Note the marked down-regulation of phospho-Akt-Ser-473 and increased levels of cleaved caspase-9 in ILK siRNA-transfected fibroblasts. B, the cells were harvested from the gels by collagenase treatment, and the levels of apoptosis in the recovered cells were quantified by TUNEL assay. Shown is the fluorescent DAPI staining (left panels) and TUNEL staining (right panels) of the recovered cells. Increased numbers of TUNEL positive cells were present in fibroblasts transfected with ILK siRNA and recovered from contractile collagen gels. C, shown is the percentage of control siRNA and ILK siRNA-transfected fibroblasts recovered from contractile collagen gels undergoing apoptosis (*, p < 0.03 versus control).
from the gels, and the level of apoptotic cells was quantified by TUNEL analysis. We found that the level of apoptosis in fibroblasts transfected with ILK siRNA and either constitutively active Akt or empty vector was 18% and 32%, respectively (Fig. 9B). In addition, the level of apoptosis in fibroblasts transfected with control siRNA and either constitutively active Akt, total Akt, and cleaved caspase-9 were determined by Western blot analysis (WB). ILK was immunoprecipitated, and ILK activity was determined by an in vitro kinase assay using a GSK3β fusion protein to assess the levels of phosphorylation of GSK3β (Ser-9). Total GSK3β was used as a loading control. Note that kinase dead ILK abrogated ILK activity, and wild type ILK increased ILK activity. Lower panels, the level of phosphorylated Akt (phospho-Akt-ser473), total Akt, and cleaved caspase-9 were determined by Western blot analysis (WB). B, upper panels, in vitro kinase assay. ILK was immunoprecipitated, and ILK activity was determined by an in vitro kinase assay using a GSK3β fusion protein to assess the levels of phosphorylation of GSK3β (Ser-9). Total GSK3β was used as a loading control. Note that kinase dead ILK abrogated ILK activity, and hyperactive ILK increased ILK activity. Lower panels, the level of phosphorylated Akt, total Akt, and cleaved caspase-9 were determined by Western analysis. C, shown is fluorescent DAPI (left panel) and TUNEL (right panel) staining of fibroblasts transfected with hyperactive ILK (HA ILK) or kinase dead ILK (KD ILK) and recovered from contractile gels at 48 h. Also shown is DAPI and TUNEL staining of untransfected cells (UN) recovered from contractile collagen gels at 48 h, to illustrate the level of apoptosis in untransfected cells. D, shown is the percentage of untransfected, kinase dead, and hyperactive ILK fibroblasts recovered from contractile gels undergoing apoptosis at 48 h (*, p < 0.05; **, p < 0.04 versus control).

**FIG. 8.** ILK activity regulates the level of Akt phosphorylation and fibroblast viability in type I collagen matrices. Human lung fibroblasts were transfected (FuGENE 6) with 4 μg of wild type ILK, hyperactive ILK (S343D), or kinase dead (E359K) ILK mutant construct. 24 h post-transfection the cells were incorporated into 0.5 mg/ml contractile collagen gels. At 48 h, the gels were either collected for Western blot analysis or dissolved with collagenase, and the recovered cells were analyzed for TUNEL staining. A, upper panels, in vitro kinase assay. ILK was immunoprecipitated (IP), and ILK activity was determined by an in vitro kinase assay using a GSK3β fusion protein to assess the levels of phosphorylation of GSK3β (Ser-9). Total GSK3β was used as a loading control. Note that kinase dead ILK abrogated ILK activity, and wild type ILK increased ILK activity. Lower panels, the level of phosphorylated Akt (phospho-Akt-ser473), total Akt, and cleaved caspase-9 were determined by Western blot analysis (WB). B, upper panels, in vitro kinase assay. ILK was immunoprecipitated, and ILK activity was determined by an in vitro kinase assay using a GSK3β fusion protein to assess the levels of phosphorylation of GSK3β (Ser-9). Total GSK3β was used as a loading control. Note that kinase dead ILK abrogated ILK activity, and hyperactive ILK increased ILK activity. Lower panels, the level of phosphorylated Akt, total Akt, and cleaved caspase-9 were determined by Western analysis. C, shown is fluorescent DAPI (left panel) and TUNEL (right panel) staining of fibroblasts transfected with hyperactive ILK (HA ILK) or kinase dead ILK (KD ILK) and recovered from contractile gels at 48 h. Also shown is DAPI and TUNEL staining of untransfected cells (UN) recovered from contractile collagen gels at 48 h, to illustrate the level of apoptosis in untransfected cells. D, shown is the percentage of untransfected, kinase dead, and hyperactive ILK fibroblasts recovered from contractile gels undergoing apoptosis at 48 h (*, p < 0.05; **, p < 0.04 versus control).
implicated in the phosphorylation of serine 473 of Akt and full activation of the kinase. ILK is present in the focal adhesion complex and is associated primarily with the β1 integrin (33–35). Nevertheless, there is no direct evidence that matrix-derived mechanical forces regulate cell viability by modulating ILK activity. We have found that when fibroblasts are incorporated into collagen matrices, they initially attach and spread, and this is associated with increases in ILK and Akt activity. However, as collagen matrix contraction ensues, the cells become progressively round, which is associated with Akt dephosphorylation and induction of apoptosis. In this report we demonstrate that ILK activity also decreases in response to collagen gel contraction down-regulates ILK activity is unclear. However, PTEN phosphatase has been shown to inhibit ILK activity. Thus, activation of specific phosphatase(s) by matrix signals might be responsible for down-regulating ILK and Akt activity. Furthermore, the mechanism by which β1 integrin-activating antibody enhances ILK activity, thereby protecting fibroblasts from collagen matrix contraction-induced apoptosis, is unclear. One possibility is antibody ligation of unligated integrin. Alternatively, collagen matrix contraction may promote alteration of the integrin conformation state from active to inactive, whereas activating antibody may facilitate maintenance of integrin in an active conformation.

The current working model for integrin outside-in signaling indicates that upon ligation of integrin by ECM, various kinases are recruited into the β1 integrin-associated focal adhesion complex (36–39). Although β1 integrin has no known intrinsic kinase function, it is feasible that ECM-β1 integrin interaction facilitates the association of ILK with β1 integrin in the focal adhesion complex, thereby activating Akt. Our immunoprecipitation assays demonstrated that upon ligation of β1 integrin with activating antibody, ILK becomes physically associated with β1 integrin. Furthermore, we found that ligation of β1 integrin by activating antibody promoted the association of ILK with Akt, and this correlated with the level of Akt phosphorylation. Taken together, these data suggest that ligation of β1 integrin by collagen or activating antibody promotes the physical association of β1 integrin, ILK, and Akt within the focal adhesion complex and the activation of the Akt survival signal.

Studies indicate that ILK may regulate cell viability by either an Akt-dependent or -independent mechanism. The above studies suggest that in our system ILK regulated fibroblast viability by modulating Akt activity. To address directly whether ILK regulates fibroblast viability by modulating Akt activity, we first knocked down ILK function using ILK siRNA; then we examined Akt activity in response to ligation of β1 integrin by type I collagen or by β1 integrin-activating antibody. Knock-down of ILK function by ILK siRNA abrogated the increase in Akt phosphorylation brought about by cell adhesion to type I collagen and by ligation of β1 integrin by β1 integrin-activating antibody. Our prior work indicates that the level of Akt activity is an important determinant governing fibroblast viability within collagen matrices (3, 11). In response to collagen matrix contraction, Akt becomes progressively dephosphorylated and fibroblasts undergo apoptosis. Therefore, we were interested in examining the effect of up- and down-regulation of ILK function on Akt activity and fibroblast survival in response to collagen matrix contraction. Both ILK siRNA and kinase dead ILK attenuated Akt phosphorylation and increased the level of fibroblast apoptosis during collagen matrix contraction. In contrast, hyperactive ILK and wild type ILK
preserved Akt activity and protected fibroblasts from collagen matrix contraction-induced apoptosis. In addition, constitutively active Akt partially reversed the decrease in Akt activity in response to ILK siRNA and effectively rescued fibroblasts from the enhanced level of apoptosis associated with ILK knock-down and collagen matrix contraction. Collectively, these data suggest that collagen contraction-derived mechanical forces are capable of modulating ILK activity, which in turn regulates fibroblast viability through an Akt-dependent mechanism. Furthermore, the mechanism by which ILK promotes phosphorylation of serine 473 of Akt is controversial. Several studies indicate that the ILK activity is directly responsible for Akt phosphorylation (13–15, 26). However, other studies suggest that ILK may function in the role of an adapter protein rather than a kinase in regulatingAkt activity (17, 18). Our results are consistent with ILK functioning as a kinase. Kinase dead ILK decreased ILK and Akt activity and augmented apoptosis, whereas hyperactive ILK increased ILK and Akt activity and protected fibroblasts from apoptosis.

Interestingly, although knock-down of ILK activity by ILK siRNA enhanced Akt dephosphorylation and augmented fibroblast apoptosis in response to contraction of type I collagen matrices, the increase in the level of apoptosis was modest. One possible explanation for this finding is that collagen matrix contraction maximally or nearly maximally decreases Akt phosphorylation and promotes apoptosis so that further down-regulation of Akt activity by ILK siRNA only modestly affects these parameters. Furthermore, because up- and down-regulation of ILK activity with subsequent alteration in Akt activity modestly affect fibroblast survival, this leaves open the possibility that an ILK/Akt-independent mechanism may also regulate fibroblast viability during collagen matrix contraction (36–40).

In summary, our working model of the molecular mechanisms regulating fibroblast viability in collagen matrices indicates that matrix-derived mechanical forces sensed by the β1 integrin are capable of modulating ILK activity downstream of PI 3-kinase, which in turn regulates the Akt viability signal. This model provides insight into the mechanism by which fibroblasts are eliminated during tissue repair.

REFERENCES
1. Desmouliere, A., Redard, M., Darby, I., and Gabbiani, G. (1995) Am. J. Pathol. 146, 56–66
2. Grimell, F., Zhu, M., Carlson, M. A., and Abrams, J. M. (1999) Exp. Cell Res. 248, 608–619
3. Tian, B., Lessan, K., Kahm, J., Kleidon, J., and Henke, C. (2002) J. Biol. Chem. 277, 24667–24675
4. Ruoslahti, E. (1996) Tumor Biol. 17, 117–124
5. Giancotti, F. G., and Ruoslahti, E. (1999) Science 285, 1028–1032
6. Dimmler, S., Assmus, B., Hermann, C., Haendeler, J., and Zeiher, A. M. (1998) Circ. Res. 83, 324–341
7. Chen, K. D., Li, Y. S., Kim, M., Li, S., Yuan, S., Chien, S., and Shyy, J. Y. (1999) J. Biol. Chem. 274, 18393–18400
8. Davies, P. F. (1995) Physiol. Rev. 75, 519–560
9. Wilson, E., Sudhir, K., and Ives, H. E. (1995) J. Clin. Investig. 96, 2364–2372
10. Ishida, T., Peterson, T. E., Kovach, N. L., and Berk, B. C. (1996) Circ. Res. 79, 310–315
11. Xia, H., Nho, R. S., Kahm, J., Kleidon, J., and Henke, C. (2004) J. Biol. Chem. 279, 32024–32034
12. Fluck, J., Querfeld, C., Cremer, A., Niland, S., Krieg, T., and Solberg, S. (1998) J. Invest. Dermatol. 110, 153–157
13. Persad, S. P., Attwell, S., Yoganathan, T. N., McPhee, T. R., Mulholland, D. J., and Dedhar, S. (2002) J. Biol. Chem. 277, 27462–27469
14. Attwell, S., Mills, J., Troussard, A., Wu, C., and Dedhar, S. (2003) Mol. Biol. Cell 14, 4813–4925
15. Persad, S., Attwell, S., Gray, V., Delcommenne, M., Troussard, A., Sanghera, J., and Dedhar, S. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 3207–3212
16. Zhang, Y., Guo, L., Chen, K., and Wu, C. (2002) J. Biol. Chem. 277, 318–326
17. Sakai, T., Li, S., Docheva, D., Grashoff, C., Sakai, K., Koutrakis G., Braun, A., Peifer, A., Yurchenco, P. D., and Fassler, R. (2003) Genes Dev. 17, 926–940
18. Lynch, D. K., Ellis, C. A., Edwards, P. A., and Hiles, I. D. (1999) Oncogene 18, 8024–8032
19. Friedman, E. B., Emerson, L., Sumita, S., Cook, D. M., Milstone, D. S., MacRae, C. A., Mariotti, M., Kuhlenkordt, P. J., Force, T., Rosenzweig, A., St-Arnaud, R., Dedhar, S., and Gerszten, R. E. (2004) Mol. Cell. Biol. 24, 8134–8144
20. Wu, C., and Dedhar, S. (2001) J. Cell Biol. 155, 505–510
21. Friedman, E. B., Sinha, S., Ling, L., Dedhar, S., Force, T., Rosenzweig, A., and Gerszten, R. E. (2002) J. Biol. Chem. 277, 16371–16375
22. Troussard, A. A., Mawji, N. M., Ong, C., Mui, A., St-Arnaud, R., and Dedhar, S. (2003) J. Biol. Chem. 278, 22374–22378
23. Frisch, S. M., and Francis, H. (1994) J. Cell Biol. 124, 619–626
24. Garaveli, Y., Sherman, Y., and Ben-Sasson, S. A. (1992) J. Cell Biol. 119, 493–501
25. Persad, S., Troussard, A. A., McPhee, T. R., Mulholland, D. J., and Dedhar, S. (2001) J. Cell Biol. 153, 1161–1174
26. Delcommenne, M., Tan, C., Gray, V., Ruel, L., Woodgett, J., and Siminovitch, K. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11121–11126
27. Zhang, X., Li, Y., Huang, Q., Wang, H., Yan, B., Dewhirst, M. W., and Li, C. Y. (2000) Clin. Cancer Res. 6, 1155–1160
28. Zhang, X., Hu, K., and Li, C. Y. (2001) Circulation 104, 2762–2766
29. Novak, A., Hsu, S. C., Leung-Hagesteijn, C., Radeva, G., Papkoff, J., Monte-sano, R., Rockelley, C., Grosschledl, R., and Dedhar, S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4374–4379
30. Cordes, N. (2004) Cancer Res. 64, 5683–5692
31. Vivanco, I., and Sawyers, C. L. (2002) Nature Rev. 2, 489–501
32. Yoganathan, T. N., Costello, P., Chen, X., Jabali, M., Yan, J., Leung, D., Zhang, Z., Yee, A., Dedhar, S., and Sanghera, J. (2000) Biochem. Pharmacol. 60, 1115–1119
33. Hannigan, G. E., Leung-Hagesteijn, C., Fitz-Gibbon, L., Copolmon, M. G., Radeva, G., Filmus, J., Bell, J. C., and Dedhar, S. (1998) Nature 393, 91–96
34. Wu, C., Knightley, S. Y., Leung-Hagesteijn, C., Radeva, G., Copolmon, M., Goicoechea, S., McDonald, J. A., and Dedhar, S. (1998) J. Biol. Chem. 273, 528–536
35. Wu, C. (1999) J. Cell Sci. 112, 4485–4489
36. Lu, Y., Yu, Q., Liu, J. H., Zhang, J., Wang, H., Koul, D., McMurray, J. S., Fang, X., Yung, W. K., Siminovitch, K. A., and Mills, G. B. (2003) J. Biol. Chem. 278, 40057–40066
37. Assoian, R. K., and Zhu, H. (1997) Curr. Opin. Cell Biol. 9, 93–98
38. Huang, Y., Li, J., Zhang, Y., and Wu, C. (2000) J. Cell Biol. 150, 861–872
39. Pankov, R., Cukierman, E., Clark, K., Matsumoto, K., Hahn, C., Poluin, B., and Yamada, K. M. (2003) J. Biol. Chem. 278, 16671–16681
40. Merlot, S., and Firtel, R. A. (2003) J. Cell Sci. 116, 3471–3478
Role of Integrin-linked Kinase in Regulating Phosphorylation of Akt and Fibroblast Survival in Type I Collagen Matrices through a β1 Integrin Viability Signaling Pathway

Richard Seonghun Nho, Hong Xia, Judy Kahm, Jill Kleidon, Deanna Diebold and Craig A. Henke

J. Biol. Chem. 2005, 280:26630-26639.
doi: 10.1074/jbc.M411798200 originally published online May 19, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M411798200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 28 of which can be accessed free at http://www.jbc.org/content/280/28/26630.full.html#ref-list-1