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Integrin-Linked Kinase Is Required for TGF-β1 Induction of Dermal Myofibroblast Differentiation

Linda Vi[1], Cristina de Lasa[1], Gianni M. DiGuglielmo[1] and Lina Dagnino[1,2]

Cutaneous repair after injury requires activation of resident dermal fibroblasts and their transition to myofibroblasts. The key stimuli for myofibroblast formation are activation of transforming growth factor-β (TGF-β) receptors and mechanotransduction mediated by integrins and associated proteins. We investigated the role of integrin-linked kinase (ILK) in TGF-β1 induction of dermal fibroblast transition to myofibroblasts. ILK-deficient fibroblasts treated with TGF-β1 exhibited attenuation of Smad 2 and 3 phosphorylation, accompanied by impaired transcriptional activation of Smad targets, such as α-smooth muscle actin. These alterations were not limited to Smad-associated TGF-β1 responses, as stimulation of noncanonical mitogen-activated protein kinase pathways by this growth factor was also diminished in the absence of ILK. ILK-deficient fibroblasts exhibited abnormalities in the actin cytoskeleton, and did not form supermature focal adhesions or contractile F-actin stress fibers, indicating a severe impairment in their capacity to differentiate into myofibroblasts. These defects extended to the inability of cells to contract extracellular matrices when embedded in collagen lattices. We conclude that ILK is necessary to transduce signals implicated in the transition of dermal fibroblasts to myofibroblasts originating from matrix substrates and TGF-β1.

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INTRODUCTION

Proper homeostasis of connective tissues, such as the dermis, depends on the ability of fibroblasts to synthesize and remodel the extracellular matrix, a process that is also critical for regeneration after wounding. On cutaneous injury, dermal fibroblasts are activated and differentiate into myofibroblasts. These cells are essential for regeneration, but can also promote pathological conditions that lead to fibrotic tissue malfunction or stroma-induced tumor progression (Hinz, 2007; Hinz *et al.*, 2007). The phenotypic changes involved in the transition of fibroblasts to myofibroblasts are well defined, and include expression of specific markers, such as α-smooth muscle actin (α-SMA), and acquisition of contractile and tissue remodeling capacity (Hinz, 2007; Hinz *et al.*, 2007). These changes occur as a consequence of multiple stimuli, the most prominent of which include activation of transforming growth factor-β (TGF-β) receptors and mechanical changes in the microenvironment (Hinz, 2010). TGF-β1 induces expression of α-SMA, extracellular matrix proteins, and other cytoskeletal factors involved in the myofibroblast contractile machinery, predominantly by Smad 2 and 3 signaling (Gu *et al.*, 2007). On the other hand, mechanotransduction is achieved mainly through integrin- and ion channel-mediated events (Katsumi *et al.*, 2004; Martinac, 2004).

Integrins interact with a number of cytoplasmic proteins, such as integrin-linked kinase (ILK), to transmit signals from the extracellular matrix to the cell. ILK is a scaffolding protein that modulates several functions relevant to tissue regeneration, including cell survival and migration, as well as actin cytoskeleton dynamics (Wickstrom *et al.*, 2010). In the skin, ILK is essential for hair follicle formation during embryogenesis and epidermal attachment to the basement membrane, as well as epidermal keratinocyte migration (Lorenz *et al.*, 2007; Nakrieko *et al.*, 2008). In spite of the large body of work conducted to elucidate the biological roles of ILK, its function in the dermis, specifically in dermal fibroblasts, remains unexplored. In this report, we address the biological role of ILK specifically in dermal fibroblasts and its response to TGF-β1, which are directly relevant to skin regeneration after injury. Our studies establish that ILK is indispensable for myofibroblast differentiation and acquisition of a contractile phenotype. Thus, ILK is a pivotal component of cellular responses necessary for normal dermal repair after wounding.

RESULTS

ILK deficiency impairs dermal fibroblast proliferation and migration

To determine the role of ILK in the dermis, we isolated and cultured primary fibroblasts from *Ilk*$^{f/f}$ mice, which allow *Ilk*

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Abbreviations: Ad, adenovirus; α-SMA, α-smooth muscle actin; βgal β-galactosidase; FBS, fetal bovine serum; FPCL, fibroblast-populated collagen lattice; ILK, integrin-linked kinase; TGF-β1, transforming growth factor-β1

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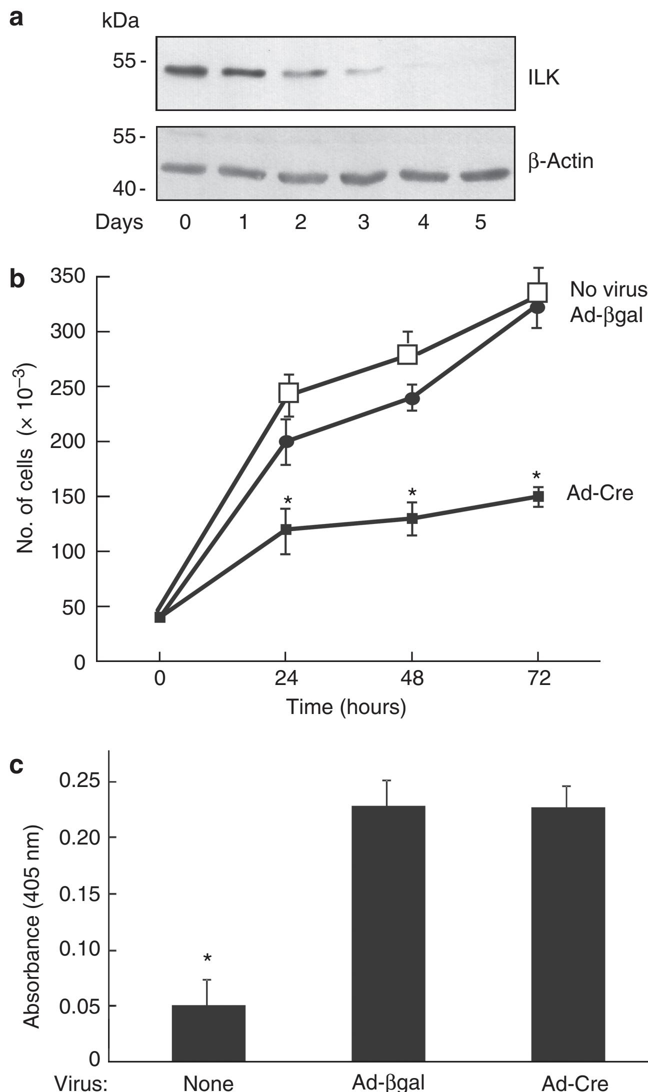

Figure 1. ILK is essential for dermal fibroblast proliferation. Primary murine dermal fibroblasts were infected with Ad-βgal or Ad-Cre, as indicated. (**a**) Protein lysates were prepared at the indicated times after infection. ILK and β-actin levels were assessed by immunoblot analysis (50 μg protein per sample). (**b**) Fibroblast numbers in the cultures were determined at the indicated times after infection. The results are expressed as mean ± SEM ($n=5$). *Indicates $P<0.05$, compared with uninfected cells (ANOVA). (**c**) Five days after adenovirus infection, lysates were prepared from fibroblast cultures containing cells attached to the culture plate and those found in the medium. The abundance of cytoplasmic mononucleosomes and oligonucleosomes was determined. The results are expressed as mean + SEM ($n=3$). *Indicates $P<0.05$ compared with Ad-βgal-treated cells (ANOVA). Ad-βgal, adenovirus encoding β-galactosidase; Ad-Cre, adenovirus encoding Cre recombinase; ANOVA, analysis of variance; ILK, integrin-linked kinase.

gene inactivation by Cre-lox recombination (Terpstra *et al.*, 2003). In fibroblasts infected with adenovirus (Ad) encoding Cre recombinase (Ad-Cre), ILK levels begin to decrease 2 days after infection, and by 3–4 days, ILK is barely detectable (Figure 1a). We next measured proliferation in uninfected cells or cells infected with Ad-Cre or β-galactosidase (Ad-βgal), as control. As shown in Figure 1b, ILK-deficient cells show decreased proliferation relative to ILK-expressing cells. To determine whether these changes are associated with decreased viability, we measured levels of mononucleosomes and oligonucleosomes (indicative of apoptosis) in these cultures. Although we observed an increase in oligonucleosome levels in infected cells, likely due to the infection step itself, >95% of the cells excluded trypan blue, indicating that they remained viable (Figure 1c and data not shown). Furthermore, no increase in apoptosis specifically due to Cre-mediated *Ilk* gene excision was evident as late as 5 days after infection, as evidenced by the similar levels of mononucleosomes and oligonucleosomes in fibroblast cultures infected with either Ad-Cre or Ad-βgal (Figure 1c). It is noteworthy that ILK-deficient fibroblast cultures began to exhibit evidence of decreased viability 6 days after the initial infection with Ad-Cre (data not shown), indicating that lack of the ILK protein over prolonged periods causes perturbations on cell functions that eventually compromise viability. For this reason, we conducted all subsequent experiments within 5 days of Ad-Cre infection, when the cells are fully viable. ILK-deficient cells also exhibited reduced capacity to adhere to and spread on laminin 1, fibronectin, and collagen 1 substrates (Supplementary Figure S1a online), and to migrate directionally in scrape-wound assays (Supplementary Figure S1b online), indicating that ILK modulates various aspects of the interactions of primary murine dermal fibroblasts with extracellular matrix substrates.

Abnormal responses to TGF-β1 in ILK-deficient dermal fibroblasts

Dermal fibroblasts respond to TGF-β1 developing phenotypic changes that signal their transition to myofibroblasts. In this and in other cell types, TGF-β receptor stimulation induces phosphorylation and activation of Smad 2 and 3, focal adhesion kinase, as well as extracellular signal-related kinase and Jun N-terminal kinase mitogen-activated protein kinases (Guo and Wang, 2009). We determined that the presence of TGF-β1 (10 ng ml^{-1}) resulted in increased phosphorylation of Smad 2 in normal fibroblast cultures, which was sustained for 24 hours. In contrast, TGF-β1 induced little, if any, increases in phosphorylated Smad 2 levels in ILK-deficient cells under the same conditions (Figure 2 and Supplementary Figure S2 online). Similarly, ILK deficiency resulted in attenuated phosphorylation of extracellular signal-related kinase and Jun N-terminal kinase upon TGF-β1 stimulation (Figure 2 and Supplementary Figure S2 online). Thus, ILK is necessary for normal activation of both canonical and noncanonical responses to TGF-β1 stimulation in dermal fibroblasts.

ILK inactivation impairs TGF-β induction of mature focal adhesions and stress fibers

The differentiation of quiescent fibroblasts into myofibroblasts induced by TGF-β1 is characterized by changes in the actin cytoskeleton, as well as by the formation of mature focal adhesions and acquisition of contractile properties. All of these changes are linked to intracellular tension (Hinz, 2007). In agreement with these properties, ILK-expressing dermal fibroblast monolayers cultured in the presence of TGF-β1

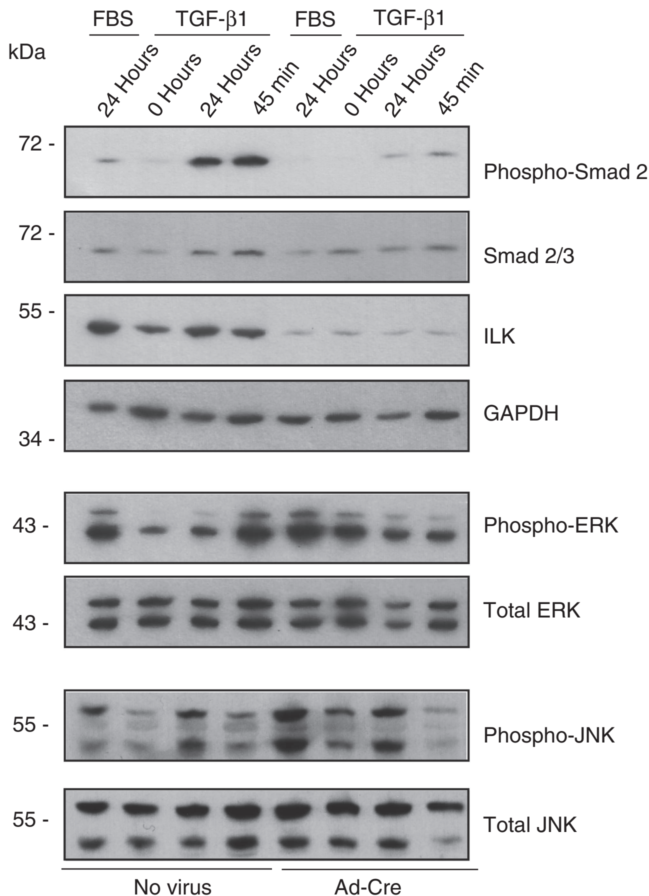

Figure 2. Abnormal Smad phosphorylation in ILK-deficient cells. Primary murine dermal fibroblasts were infected with Ad-Cre and cultured for 48 hours in normal growth medium. The cells were then cultured in either fresh medium containing 8% FBS (FBS) or 1% BSA for 24 hours, followed by culture in medium containing either 8% FBS (FBS) or TGF-β1 (10 ng ml^{-1}). Cell lysates were prepared at the indicated times after TGF-β1 addition, and analyzed by immunoblot using antibodies against the indicated proteins. Anti-Smad antibodies react with both Smad 2 and 3. Levels of GAPDH were used to normalize for loading. The blot shown is representative of six different experiments. Ad-Cre, adenovirus encoding Cre recombinase; ERK, extracellular signal-related kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphodehydrogenase; ILK, integrin-linked kinase; TGF-β1, transforming growth factor-β1.

developed large, ''supermature'' focal adhesions, as evidenced by vinculin-associated immunofluorescence, irrespective of whether the cells were infected with Ad-βgal (Figure 3a). The presence of fetal bovine serum (FBS) in these cultures also induced focal adhesions, although they were somewhat less developed (Figure 3a). In contrast, the focal adhesions formed in ILK-deficient fibroblasts in response to TGF-β1 were substantially smaller and less organized (Figure 3a). A hallmark of myofibroblast differentiation is the upregulation of α-SMA and its incorporation into the actin cytoskeleton, which are events triggered by TGF-β1 and mechanical tension. Given that ILK modulates actin cytoskeleton remodeling, which is a key function for the formation of focal adhesions, we also examined the status of α-SMA and F-actin in these cells. Both proteins were readily detected in the abundant, thick stress fibers organized along the cell body in ILK-expressing fibroblasts treated with either FBS or TGF-β1 (Figure 3b). In contrast, scarce α-SMA and actin were found in thin and disorganized filaments in ILK-deficient cells (Figure 3b). This suggests a link between the absence of ILK and defective formation of supermature focal adhesions, aberrant F-actin remodeling, and incorporation of α-SMA in stress fibers in response to TGF-β1.

Transcriptional responses to TGF-β1 require ILK

The altered F-actin cytoskeleton in ILK-deficient fibroblasts is unlikely to be caused by changes in β-actin abundance, as its levels in the presence or absence of ILK are similar (Figure 1a). Thus, we next examined whether α-SMA upregulation by TGF-β1 treatment is normal in ILK-deficient cells cultured as monolayers. Remarkably, neither TGF-β1 nor FBS treatment induced α-SMA protein upregulation in the absence of ILK (Figure 4a). The *α-SMA* gene is transcriptionally activated by TGF-β1 by Smad activation. Primary murine dermal fibroblasts transfect very poorly, thus precluding the development of reporter assays to measure Smad-directed transcription. Thus, to determine whether reduced Smad phosphorylation in ILK-deficient cells is associated with attenuated transcriptional responses to TGF-β1, the status of plasminogen activator inhibitor-1, another well-established TGF-β1 target regulated through Smad-dependent transcription (Eitzman *et al.*, 1996), was examined. Similar to α-SMA, TGF-β1 treatment failed to increase plasminogen activator inhibitor-1 levels in ILK-deficient cells (Supplementary Figure S3a online), consistent with the notion that ILK is required to transduce Smad-modulated transcriptional responses to TGF-β1 in fibroblasts.

The differentiation of fibroblasts into myofibroblasts in a three-dimensional context is also tightly regulated by the sum of chemical and mechanical stimuli (Hinz, 2010). Hence, we extended our analysis to assess changes in α-SMA expression in fibroblasts embedded in collagen lattices. For these experiments, we used uninfected fibroblasts, or cells infected with either Ad-βgal or Ad-Cre. Three days after Ad infection, cells were briefly trypsinized and embedded in collagen gels in the presence or absence of TGF-β1 (10 ng ml^{-1}). The gels were overlaid with growth medium in the presence or absence of TGF-β1 for 24 hours, and then released for up to 24 additional hours (i.e., total time of 5 days after Ad-Cre infection, thus ensuring cell viability). α-SMA protein levels in ILK-expressing cells showed a marked increase in the presence of TGF-β1 (Figure 4b), consistent with the notion that myofibroblasts are generated under these conditions. In contrast, no upregulation of the α-SMA protein in response to TGF-β1 was detected in lysates from fibroblast-populated collagen lattices (FPCLs) prepared with ILK-deficient fibroblasts (Figure 4b). Furthermore, quantitative real-time-PCR analysis revealed that ILK deficiency precluded increases in α-SMA mRNA in response to TGF-β1, which were readily detected in ILK-expressing cells (Figure 4c). Similar to our observations with fibroblast monolayers, plasminogen activator inhibitor-1 protein levels were increased in ILK-expressing, but not in ILK-deficient, fibroblasts embedded

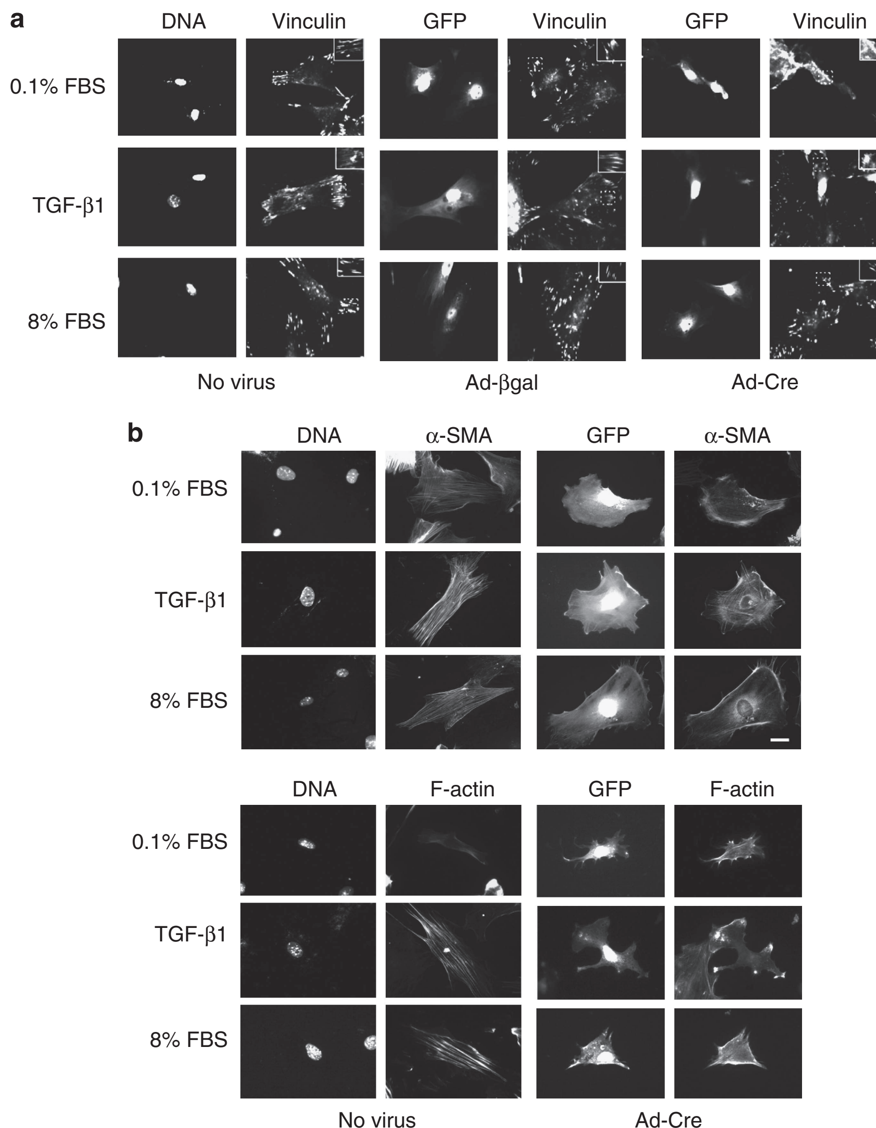

Figure 3. ILK-deficient cells exhibit abnormal F-actin cytoskeleton and focal adhesions. Primary dermal fibroblasts were infected with the indicated adenovirus and cultured for 72 hours before processing for fluorescence microscopy. (**a**) Focal adhesions were visualized with antibodies against vinculin. The actin cytoskeleton was visualized with (**b**) antibodies against α-SMA or (**c**) with Alexa 594-conjugated phalloidin. Adenovirus-infected cells were identified by GFP fluorescence, and DNA was visualized with Hoescht 33342. Insets are higher magnification images of boxed areas. Bar = 25 μm. Ad-βgal, adenovirus encoding β-galactosidase; Ad-Cre, adenovirus encoding Cre recombinase; FBS, fetal bovine serum; GFP, green fluorescent protein; ILK, integrin-linked kinase; α-SMA, α-smooth muscle actin; TGF-β1, transforming growth factor-β1.

in collagen lattices (Supplementary Figure S3b online). Taken together, these observations suggest that ILK is essential for fibroblast transition to myofibroblasts, and that ILK is a necessary element in TGF-β receptor activation of Smad-regulated responses in these cells.

Role of ILK in myofibroblast contractility

Increases in α-SMA and stress fibers in response to TGF-β1 are important for the contractile properties of myofibroblasts. As a model of a three-dimensional wound environment, dermal fibroblasts were embedded in collagen matrices attached to the culture plate to generate FPCLs in the presence of TGF-β1.

Myofibroblasts in FPCLs containing TGF-β1 exhibited a polarized morphology with pseudopodial extensions and thick actin stress fibers arranged along the cell body, and often decorating cell protrusions (Figure 5a). In stark contrast, ILK-deficient fibroblasts appeared either more elongated with numerous dendritic processes (Figure 5a) or rounded with a few dendritic extensions (Figure 5b). Irrespective of their morphology, ILK-deficient cells showed few, if any, F-actin bundles (Figure 5a). Similarly, the presence of FBS in FPCLs induced formation of stress fibers in ILK-expressing, but not in ILK-deficient, cells (Figure 5a), thus indicating that ILK is also essential for normal actin cytoskeleton formation and organization in a three-dimensional context.

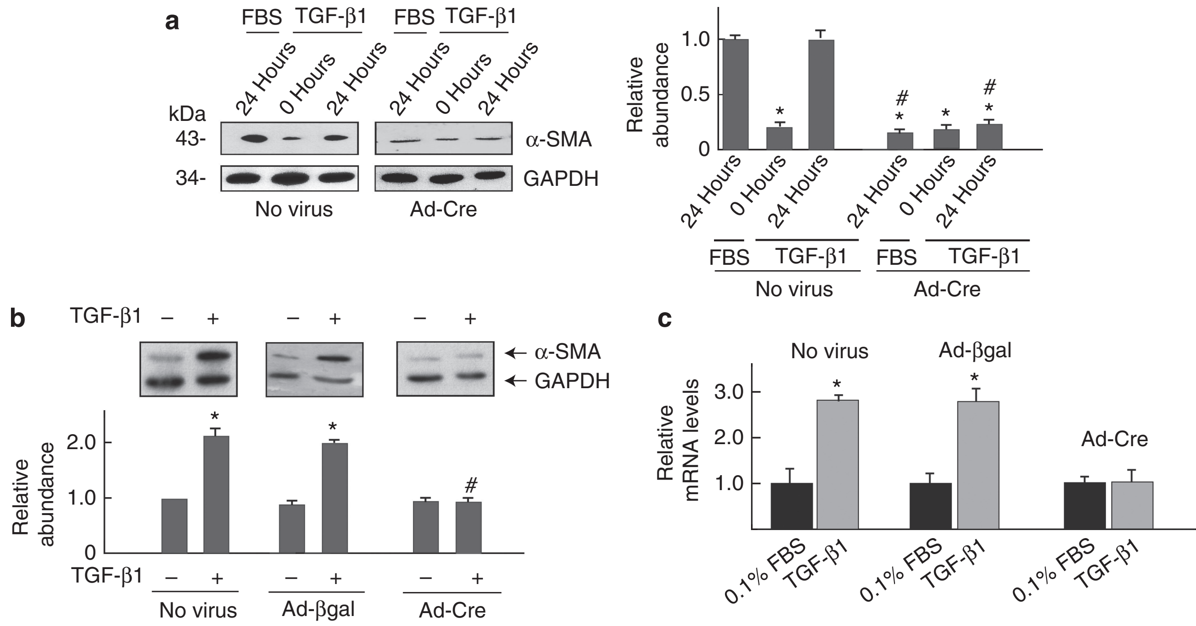

Figure 4. Impaired regulation of α-SMA by TGF-β1 in ILK-deficient dermal fibroblasts. Dermal fibroblasts were infected with the indicated adenoviruses, and cultured for 72 hours before changing to the following conditions: (**a**) Cells were cultured in medium containing 0.1% FBS for 16 hours, and switched to medium containing 8% FBS or TGF-β1 (10 ng ml^{-1}, final). Cell lysates were prepared at the indicated times after TGF-β1 addition and were analyzed by immunoblot with antibodies against α-SMA or GAPDH. A representative blot is shown. The histogram represents densitometric analysis of blots from three separate experiments, and the average normalized abundance (+SD) is shown. The results are expressed relative to α-SMA levels in noninfected cells treated with FBS, set to 1. *Indicates $P<0.05$ relative to α-SMA levels in noninfected cells treated with FBS, and #indicates $P<0.05$ relative to the corresponding sample in noninfected fibroblasts (ANOVA). (**b**) Fibroblasts were briefly trypsinized and used to generate FPCLs containing 0.1% FBS or TGF-β1 (10 ng ml^{-1}, final). The gels were cultured for 24 hours, and then were released to induce contraction for 24 additional hours, and were processed to obtain protein lysates, which were analyzed by immunoblot with the indicated antibodies. A representative blot is shown. The histogram represents densitometric analysis of blots from three separate experiments and the average normalized abundance (+SD) is shown. The results are expressed relative to α-SMA levels in noninfected cells treated with FBS, set to 1. *Indicates $P<0.05$ relative to α-SMA levels in noninfected cells treated with FBS, and #indicates $P<0.05$ relative to the corresponding sample in noninfected fibroblasts. (**c**) FPCLs were obtained as described in panel **b**, but were processed to isolate RNA for real-time PCR analysis to assess α-SMA transcript levels 8 hours after release. The data are normalized to control 18S RNA, and are expressed relative to uninfected cells in 0.1% FBS-containing gels, set to 1. Results are expressed as mean+SEM ($n=3$). *Indicates $P<0.05$ compared with uninfected cells in 0.1% FBS-containing gels (ANOVA). Ad-βgal, adenovirus encoding β-galactosidase; Ad-Cre, adenovirus encoding Cre recombinase; ANOVA, analysis of variance; FBS, fetal bovine serum; FPCL, fibroblast-populated collagen lattice; GAPDH, glyceraldehyde-3-phosphodehydrogenase; ILK, integrin-linked kinase; α-SMA, α-smooth muscle actin; TGF-β1, transforming growth factor-β1.

The interaction of myofibroblasts with surrounding collagen in the extracellular matrix involves binding through focal adhesions and remodeling of collagen fibrils, which lead to contraction through mechanical forces (Kim *et al.*, 2006; Dahlmann-Noor *et al.*, 2007). We assessed the patterns of collagen fibril organization by ILK-expressing and ILK-deficient cells in the presence of TGF-β1, using confocal reflection microscopy. This approach allows to directly visualize cell–matrix interactions through the overlay of reflected light images of collagen fibrils and the F-actin network in fibroblasts (Kim *et al.*, 2006). In ILK-expressing myofibroblasts, which were either left uninfected or previously infected with Ad-βgal, collagen was visualized in the form of compacted fibrils aligned parallel to the cell body and frequently concentrated around cell extensions (Figure 5b). In contrast, collagen fibrils adjacent to ILK-deficient cells were frequently randomly oriented and less compacted, indicative of impaired interactions between collagen fibers and cells (Figure 5b).

We next examined the functional consequences of the abnormal responses to TGF-β1 and cell–matrix interactions observed in the absence of ILK. We generated FPCLs containing ILK-expressing fibroblasts (either not infected or infected with Ad-βgal) or ILK-deficient fibroblasts (infected with Ad-Cre) cultured in the presence or absence of TGF-β1. The ability of cells to contract the gels after release was then measured. As before, and to conduct these assays within the period during which cells remain viable, we infected fibroblasts with Ad-βgal or Ad-Cre, and 3 days later, we used these cells to generate FPCLs. The lattices were cultured for 24 hours in the presence or absence of TGF-β1 and were then released. We measured the surface area of these gels at timed intervals after release, as an estimate of cell-mediated contraction. Given that the presence of TGF-β1 under these conditions stimulated α-SMA expression in normal cells, which defines the formation of myofibroblasts, we reasoned that this approach would allow us to determine the consequences of *Ilk* inactivation on the contractile capacity of cells. Contraction of the matrix by uninfected myofibroblasts, or cells infected with Adβ-gal was evident within 10 minutes of release, and continued for several hours (Figure 6). The surface contraction in FPCLs containing uninfected fibroblasts reached maximum levels of 60±4%. The decrease in gel surface in FPCLs with Ad-βgal-infected fibroblasts was slightly lower, reaching

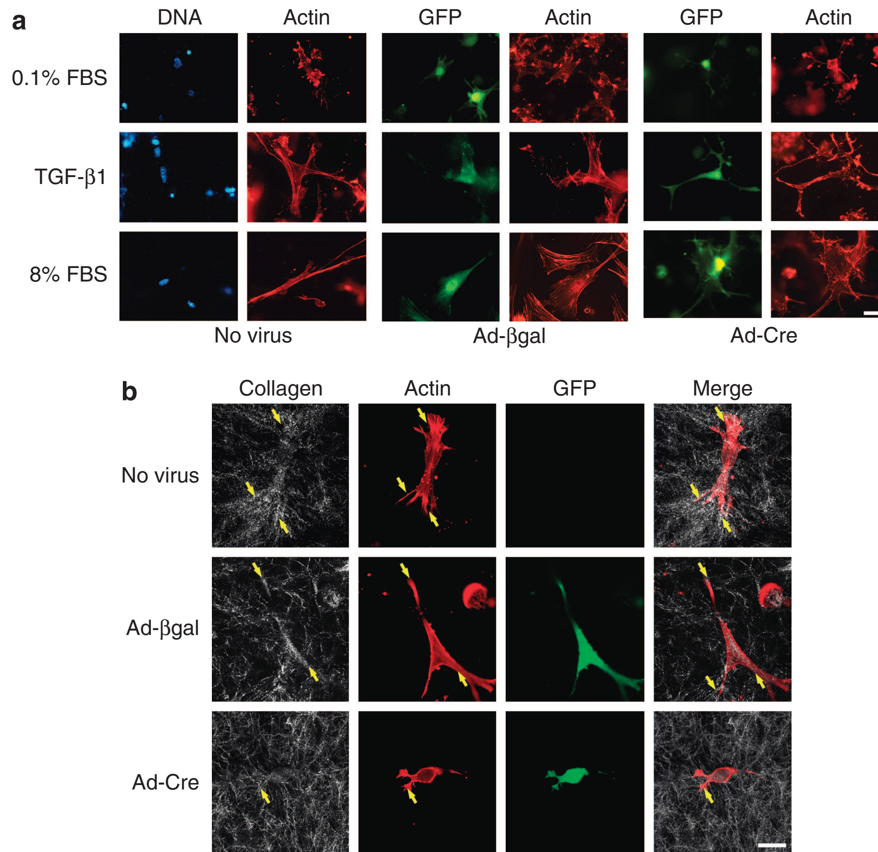

Figure 5. Abnormalities in F-actin cytoskeleton and collagen remodeling in ILK-deficient cells. (**a**) Fibroblasts infected with Ad-βgal or Ad-Cre were used to generate FPCLs containing FBS or TGF-β1 (10 ng ml^{-1}, final). The gels were incubated for 24 hours and released for 8 hours before processing for fluorescence microscopy. DNA and F-actin were visualized with Hoescht 33432 and Alexa 594-conjugated phalloidin, respectively. Infected cells were identified by GFP fluorescence. (**b**) Fibroblasts were infected and cultured in FPCLs containing TGF-β1 (10 ng ml^{-1}) for 24 hours. FPCLs were processed for confocal microscopy, using Alexa 594-conjugated phalloidin and GFP to detect infected cells. Collagen fibrils in the gel were detected by reflection microscopy. Arrows indicate regions with cell protrusions. Bar = 25 μm. Ad-βgal, adenovirus encoding β-galactosidase; Ad-Cre, adenovirus encoding Cre recombinase; FBS, fetal bovine serum; FPCL, fibroblast-populated collagen lattice; GFP, green fluorescent protein; ILK, integrin-linked kinase; TGF-β1, transforming growth factor-β1.

55 ± 3% by 24 hours (Figure 6). In stark contrast, minimal contraction was observed in TGF-β1-treated ILK-deficient FPCLs, which was indistinguishable from that measured in the absence of this cytokine (Figure 6). Taken together, these observations indicate that the absence of ILK results in impaired fibroblast mechanotransduction, generation of contractile capacity, and transition to a myofibroblasts phenotype in response to TGF-β1.

DISCUSSION

Although it is clear that myofibroblast differentiation requires multiple coordinated events (Hinz, 2010), the molecular mechanisms that operate during this process are not fully understood. Our studies demonstrate that ILK is essential for myofibroblast differentiation through modulation of TGF-β signaling.

The major inducers of myofibroblast differentiation are TGF-β1 stimulation and integrin-mediated mechanotransduction (Hinz, 2010), and our findings suggest that ILK may function to integrate these two processes in dermal fibroblasts. Signaling through Smad is a prominent mechanism for TGF-β induction of myofibroblast formation. A key observation in our studies is that ILK is essential for Smad-mediated responses to TGF-β1 in murine dermal fibroblasts. Indeed, in the absence of ILK, TGF-β1 stimulation failed to induce normal Smad 2 phosphorylation and upregulation of α-SMA. Reporter assays to further confirm impaired transcriptional activation by Smad were not feasible in these cells because of their poor transfection efficiency. However, TGF-β1 treatment did not increase levels of plasminogen activator inhibitor-1, another Smad transcriptional target. Taken together, the results suggest a modulatory role for ILK downstream of the TGF-β receptor, but upstream of Smad-dependent transcription, which to our knowledge is previously unreported. This relationship differs from the reported upregulation of ILK by TGF-β1 in renal tubular cells undergoing epithelial–mesenchymal transition (Li *et al.*, 2003).

The precise mechanisms involved in ILK regulation of Smad activation remain to be elucidated. Integrins and TGF-β are known to crosstalk in fibroblasts. For example, integrins are necessary for activation of latent TGF-β1 and can amplify TGF-β signaling through physical interaction with its receptors (Margadant and Sonnenberg, 2010). TGF-β1stimulation can also promote interactions between its type II receptor and

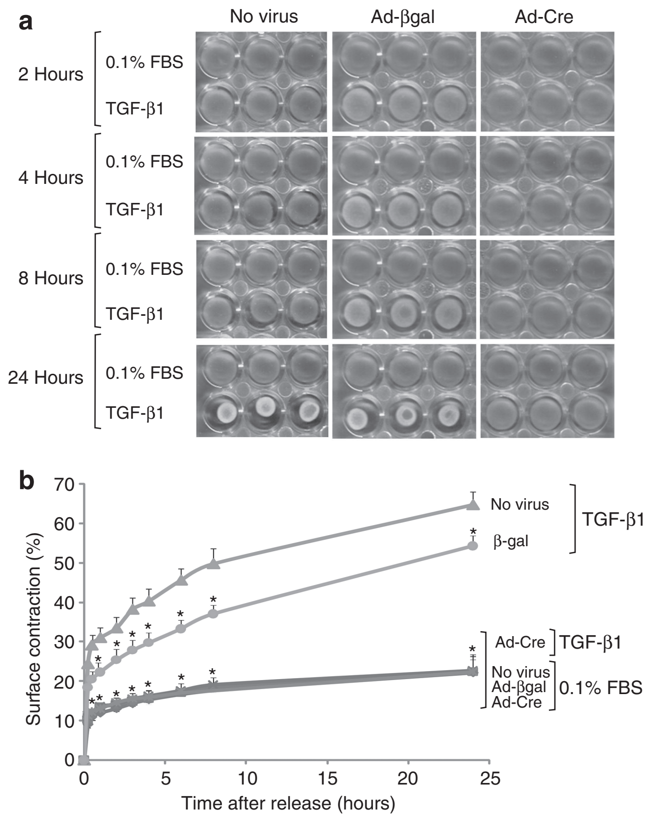

Figure 6. Impaired contractile properties of ILK-deficient fibroblasts. (**a**) Fibroblasts infected with adenovirus encoding GFP and the indicated proteins were used to generate FPCLs containing 0.1% FBS or TGF-β1 (10 ng ml^{-1}, final). The gels were incubated for 24 hours, before being released. Images were acquired at the indicated times after gel release. (**b**) Contraction of the FPCLs described in panel **a** was quantified, and is expressed as the percentage of the gel surface area at $t=0$ (time of release). The results are expressed as mean + SEM ($n=3$). *Indicates $P<0.05$ relative to contraction in uninfected cultures (repeated-measures ANOVA). Ad-βgal, adenovirus encoding β-galactosidase; Ad-Cre, adenovirus encoding Cre recombinase; ANOVA, analysis of variance; FBS, fetal bovine serum; FPCL, fibroblast-populated collagen lattice; GFP, green fluorescent protein; ILK, integrin-linked kinase; TGF-β1, transforming growth factor-β1.

several αvβ integrins in lung and scleroderma fibroblasts. In the latter cell type, focal adhesion kinase activation through integrins is necessary for TGF-β induction of myofibroblast differentiation (Scaffidi *et al.*, 2004; Asano *et al.*, 2006). Furthermore, collagen and other matrix molecules can induce the association of integrin α2β1 with types I and II TGF-β receptor oligomers, forming a signaling complex that also includes activated focal adhesion kinase and Smad 2/3 phosphorylation (Scaffidi *et al.*, 2004; Asano *et al.*, 2006). It is conceivable that optimal function of these signaling complexes also requires ILK, a concept that is an important area for future research. Of potential relevance to this concept is the observed requirement for ILK in mechanotransduction signals that activate focal adhesion kinase in certain tumor cells (Wang and Basson, 2009). The involvement of ILK in the regulation of TGF-β signaling in dermal fibroblasts is a function for this scaffold protein that may be independent of its previously identified activities.

Integrin-linked kinase is necessary for dermal fibroblast adhesion to the extracellular matrix, forward movement on two-dimensional substrates, and actin cytoskeleton dynamics, functions that it also fulfills in other epithelial and mesenchymal cell types (Qian *et al.*, 2005; Nakrieko *et al.*, 2008). In addition, our data are consistent with a role for ILK as a transducer of cell interactions with the surrounding microenvironment and ability to remodel extracellular collagen fibrils in a three-dimensional context, which has important potential implications for tissue remodeling *in vivo*. Significantly, ILK-deficient fibroblasts cultured on rigid substrates as monolayers do not develop prominent stress fibers, a characteristic of protomyofibroblasts. The latter are cells that represent the first phase in myofibroblast generation. ILK-deficient cells do not appear to develop further into myofibroblasts that express high levels of α-SMA either, even after stimulation with TGF-β1. Taken together, these observations suggest that ILK regulates the transition of fibroblasts to myofibroblasts, likely at multiple levels, including mechanotransduction by integrins and signaling through the TGF-β/Smad pathways. ILK deficiency also affected the stimulation of mitogen-activated kinases by TGF-β, suggesting a more generalized decrease in cell responses to myofibroblast-inducing stimuli. The key areas for future research are the elucidation of mechanisms involved in the regulation by ILK of TGF-β1-induced responses in dermal fibroblasts, and the consequences of manipulating ILK function on skin regeneration and fibrosis *in vivo*.

MATERIALS AND METHODS

Cell culture and adenoviral infections

Primary dermal fibroblasts were isolated from mice containing *Ilk* alleles flanked by loxP sequences (*Ilk*$^{f/f}$ mice; Terpstra *et al.* (2003)). The skin of 3-day-old mice was harvested and digested overnight with trypsin as described previously (Ho *et al.*, 2009; Ivanova *et al.*, 2009). After trypsin digestion, the epidermis was removed, and the dermis was minced and further digested with a solution containing 0.35% collagenase type I dissolved in HyQ-DMEM-RS (HyClone, Fisher Scientific, Whitby, Ontario, Canada). Cell debris and undigested tissue were removed by filtration through 100-μm filters (Falcon, BD Biosciences, Bedford, MA), and cells were centrifuged, resuspended in culture medium consisting of HyQ-DMEM-RS supplemented with 8% FBS, 1% each penicillin G, and streptomycin (Invitrogen, Grand Island, NY), and seeded on culture dishes. All experiments were conducted on cells that had reached ⩽80% confluence and had been passaged 1–4 times. For viral infections, fibroblasts were incubated with the appropriate Ad at a multiplicity of infection of 100–150 for 4 hours in serum-fee HyQ-DMEM-RS, followed by additional culture for 72–96 hours in HyQ-DMEM-RS containing 8 or 0.1% FBS/0.05% bovine serum albumin, as indicated in individual experiments. Infections consistently yielded ⩾95% transduction efficiency without significant cytotoxicity. The recombinant Ads used encode either green fluorescent protein and β-gal (Ad-βgal) (Nakrieko *et al.*, 2008) or green fluorescent protein and Cre recombinase (Ad-Cre, Vector Biolabs, Philadelphia, PA).

FPCL contractility assays

Collagen lattices were polymerized in 24-well tissue culture trays. Dermal fibroblasts were mixed with a solution of collagen I (BD

Biosciences, Bedford, MA), to yield a final volume of 500 μl containing 100,000 cells, 1.2 mg ml^{-1} collagen, and either 10 ng ml^{-1} TGF-β1 or vehicle (ddH$_2$O). The cell/collagen suspension was incubated at 37 °C for 25 minutes to allow the mixture to gel, followed by gentle addition of 1 ml of HyQ-DMEM-RS supplemented with either 0.1% FBS/0.05% bovine serum albumin or TGF-β1 (10 ng ml^{-1})/0.05% bovine serum albumin. The gels were cultured for 24 hours, at which time they were mechanically released. Digital images of the floating lattices were captured at timed intervals up to 24 hours after release. To quantify contraction, the FPCL surface area was determined using the freehand tool in ImageJ software (NIH, Bethesda, MD), and sequential area calculations were normalized to the area measured immediately after release. All experiments were performed at least three times with triplicate samples. Statistical analyses were conducted using analysis of variance with *post hoc* Bonferroni's correction.

Confocal reflection microscopy

Cells were cultured in FPCLs as described above for 24 hours. To prepare for microscopy, FPCLs were fixed with 4% paraformaldehyde in phosphate-buffered saline for 1 hour and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 20 minutes. F-actin and DNA were visualized with Alexa 594-conjugated phalloidin and Hoescht 33342, respectively. After staining, the gels were transferred to glass-bottom culture dishes (MatTek, Ashland, MA) for analysis using confocal reflection microscopy as described previously (Voytik-Harbin *et al.*, 2001), using a Zeiss LSM5 DuoVarioII scanning laser confocal microscope (Zeiss, Jena, Germany) equipped with a ×63/1.4 NA oil immersion lens. Specimens were visualized with 633, 543, and 488 nm lasers, using Zen 2009 software (Zeiss).

Quantitative real-time PCR

Total RNA from cells cultured in FPCLs was isolated using RNeasy Plus Micro kits (Qiagen, Mississauga, Ontario, Canada), following the protocol for isolation of RNA from animal tissues recommended by the manufacturer. RNA quality was determined on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA), and 25 ng of high-quality RNA for each sample was subjected to reverse transcription and amplification using TaqMan Assays on Demand (Applied Biosystems, Streetsville, Ontario, Canada). The primer/probe sets used to determine relative transcript abundance were α-α-SMA (ACTA2Mm 00725412_s1) and eukaryotic 18S rRNA (4352930E). Amplified sequences were detected using a Prism 7900HT sequence detector (Applied Biosystems). Synthesis of cDNA templates was conducted at 48 °C for 30 minutes, followed by 40 cycles of amplification (95 °C for 15 seconds; 60 °C for 1 minute). The results were analyzed using SDS v2.1 software (Applied Biosystems). Relative α-SMA transcript levels were calculated using the ΔΔCt method.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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