

# Focal Adhesion Kinase (FAK)-related Non-kinase Inhibits Myofibroblast Differentiation through Differential MAPK Activation in a FAK-dependent Manner\*

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Transforming growth factor (TGF)- $\beta$ 1 induces fibroblast transdifferentiation to myofibroblasts, a process that requires the involvement of integrin-mediated signaling and focal adhesion kinase (FAK). FAK-related non-kinase (FRNK) is known for its role in inhibiting integrin-mediated cell migration; however, its role in myofibroblast differentiation has not been defined. Here, we report that FRNK abrogates TGF- $\beta$ 1-induced myofibroblast differentiation *in vitro* and *in vivo*. TGF- $\beta$ 1 can induce  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) expression in the presence or absence of FAK; however, TGF- $\beta$ 1-induced  $\alpha$ -SMA expression is reduced (~73%) in FAK-deficient fibroblasts. Although both ERK and p38 MAPK activation is required for maximal TGF- $\beta$ 1-induced  $\alpha$ -SMA expression, ERK is the major signaling intermediate in cells that express FAK. In contrast, p38 MAPK is the dominant mediator of TGF- $\beta$ 1-induced  $\alpha$ -SMA expression in FAK-deficient cells. FRNK overexpression blocks TGF- $\beta$ 1-induced ERK or p38 MAPK activation in the presence, and surprisingly, in the absence of FAK. The loss of FRNK was tested *in vivo* during experimentally induced pulmonary fibrosis in mice. FRNK knock-out mice have a greater increase in  $\alpha$ -SMA-expressing cells in response to a pulmonary fibrotic stimulus *in vivo*, as compared with congenic wild type mice. This is the first time that FRNK loss has been shown to modify the pathobiology in any animal disease model. Together, the data demonstrate that FRNK negatively regulates myofibroblast differentiation *in vitro* and *in vivo*. These data further suggest that modulation FRNK expression may be a novel avenue for therapeutic intervention in tissue fibrosis.

Deranged tissue repair and remodeling or failed termination of the normal wound healing response may result in tissue fibrosis. After injury, fibroblasts migrate into the wound, proliferate, and differentiate to “activated” fibroblasts (1–4). The

differentiated or activated fibroblasts are termed myofibroblasts and are present in abundance within fibrotic lesional areas in the lung, liver, and kidney (2, 4–6). Myofibroblasts secrete profibrotic cytokines and extracellular matrix proteins, such as collagen and fibronectin, and therefore have been presumed to act as effector cells of the fibrotic process (1, 2, 4–7).

Idiopathic pulmonary fibrosis is a devastating, untreatable, progressive fibrotic disease of the lung (8–11). In support of the importance of myofibroblasts in the pathogenesis of lung fibrosis, these cells localize to fibroblastic foci in pulmonary fibrotic lungs, and the extent of fibroblastic foci is a major prognostic factor for patients with idiopathic pulmonary fibrosis (8–11).

The phenotypic hallmarks of myofibroblasts are the expression of  $\alpha$ -SMA<sup>2</sup> and the incorporation of  $\alpha$ -SMA into well organized cytoplasmic fibers (1, 4, 6). These fibers connect to robust, mature focal adhesions (1).  $\alpha$ -SMA expression is associated with an enhanced contractile phenotype and can directly enhance the contractility of fibroblasts (12, 13). Inhibition of  $\alpha$ -SMA integration into cytoplasmic fibers by  $\alpha$ -SMA N-terminal peptides blocks cell contraction and collagen expression in rat lung fibroblasts (14).  $\alpha$ -SMA-deficient mice have impaired vascular contractility and show increased renal fibrotic lesions in a unilateral ureteral obstruction model (15, 16). These data indicate that  $\alpha$ -SMA plays a key role in regulation of myofibroblast functions *in vitro* and *in vivo*.

Although the myofibroblast is generally accepted as a key effector cell in the development of fibrosis, the molecular mechanisms and intracellular signaling events regulating myofibroblast differentiation are not completely understood. TGF- $\beta$  is the best studied positive regulator of myofibroblast differentiation (17–19) and is widely accepted as a central mediator of the fibrotic responses in lung, liver, and kidney (5, 8, 20). Myofibroblast differentiation is dependent on the presence of active TGF- $\beta$ , a rigid extracellular matrix, and the presence of the extra type III domain - A (EDA) fibronectin fragment (1, 21–23). Integrin receptors are responsible for

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<sup>2</sup> The abbreviations used are:  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; FAK, focal adhesion kinase; FRNK, FAK-related non-kinase; Ad-, adenoviral construct; TGF, transforming growth factor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; HA, hemagglutinin; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; TLCK, *N*<sup>α</sup>-*p*-tosyl-L-lysine chloromethyl ketone; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GFP, green fluorescent protein; Luc, luciferase; BSA, bovine serum albumin; MOI, multiplicity of infection; MEK, MAPK/ERK kinase.

## FRNK Inhibits Myofibroblast Differentiation

transmitting these matrix environmental cues intracellularly (24, 25). In fact, integrins can participate in the activation of matrix-bound latent TGF- $\beta$  through cytoskeletal-induced generation of tension (21, 26).

Integrin engagement with extracellular matrix proteins can activate focal adhesion kinase (FAK) and results in an increased phosphorylation of Tyr<sup>397</sup> of FAK (25, 27–30). FAK has been demonstrated to have a critical role in integrin-mediated cell functions, such as cell migration and proliferation (25, 31–33). FAK has recently been shown to mediate the  $\alpha$ -SMA up-regulation in response to TGF- $\beta$ 1 (34, 35), the  $\alpha$ -SMA inhibition in response to fibroblast growth factor (36).

FRNK is an independently expressed cytoplasmic protein that is identical in sequence to the C-terminal region of FAK (25, 37, 38). It has been demonstrated that FRNK inhibits integrin-mediated FAK activation (Tyr<sup>397</sup> phosphorylation) and blocks FAK-mediated cell migration and proliferation (25, 37, 39, 40). However, the role of FRNK in TGF- $\beta$ 1-induced myofibroblast differentiation has not been defined.

In this study, the effect of gain of FRNK and the FAK dependence of the effect of FRNK on TGF- $\beta$ 1-induced  $\alpha$ -SMA expression were examined. Furthermore, because MAPKs are known downstream mediators of FAK signaling (41–43), we studied the FAK dependence of MAPK activation during myofibroblast differentiation. Lastly, we evaluated the effect of FRNK deficiency on myofibroblast differentiation in a murine model of experimentally induced pulmonary fibrosis.

### EXPERIMENTAL PROCEDURES

**Reagents**—TGF- $\beta$ 1 was obtained from R & D Systems (Minneapolis, MN). The following purified polyclonal antibodies were purchased: anti-phospho-FAK (Tyr(P)<sup>397</sup>) (BIOSOURCE, Camarillo, CA), anti-FAK (recognizes both FAK C-terminal and FRNK; Upstate Biotechnology, Lake Placid, NY), anti-phospho-ERK (Cell Signaling Technology/New England Biolabs, Danvers, MA), anti-ERK IgG (Santa Cruz Biotechnology, Santa Cruz, CA), anti-p38 (Cell Signaling Technology), anti-phospho-p38 (Chemicon International, Temecula, CA), and anti-phospho-JNK (Cell Signaling). Purified Alexa Fluor 488 goat anti-mouse IgG was purchased from Molecular Probes (Carlsbad, CA). The following purified monoclonal antibodies were purchased: anti- $\alpha$ -SMA (American Research Products, Belmont, MA), Cy3-conjugated anti- $\alpha$ -SMA antibody (clone 1A4; Sigma-Aldrich), anti-hemagglutinin (HA) epitope tag (Santa Cruz Biotechnology), and anti-glyceraldehyde 3-phosphate dehydrogenase (G3PDH) (Research Diagnostics, Flanders, NJ). The selective inhibitors PD98059, U0126, U0124, and SB203580 were purchased from EMD Biosciences/Calbiochem and dissolved in Me<sub>2</sub>SO.

**Animal Protocol**—Animal interventions were approved by the Institutional Animal Care and Use Committee and the Animal Resources Facility at the University of Alabama at Birmingham and were performed under the guidelines for animal care recommended by the American Physiological Society. All of the animals were pathogen-free, housed in micro-isolator cages, and fed with autoclaved food and water. FRNK knock-out mice were generated as described (44) and were generously provided by Dr. J. Thomas Parsons. FRNK knock-out mice were C57BL/6

background (back-crossed with C57BL/6 mice for at least 12 generations before the current study). The genotype of FRNK knock-out (homozygous) mice and their wild type littermates was confirmed by PCR using genomic DNA extracted from tails as published (44), and the absence of FRNK expression in lung tissue from FRNK knock-out mice was confirmed by Western blot (data not shown). The administration of bleomycin and harvest of lung tissues were described previously (45, 46). Briefly, the animals (7–10 weeks) were anesthetized, and bleomycin (1 unit/kg of body weight in 50  $\mu$ l of saline) or saline alone (50  $\mu$ l) was slowly instilled through airway to lungs by using an intratracheal catheter. The skin wounds were closed, and the animals were allowed to recover. The animals were euthanized at day 18 after bleomycin instillation. The lungs were perfused with cold phosphate-buffered saline, inflated with tissue-preservation media Optimal Cutting Temperature compound (OCT), and sectioned for immunofluorescence analysis of  $\alpha$ -SMA-positive cells. Whole lung homogenate supernatants were used for Western blot analysis. The harvested lungs were homogenized in 1% Nonidet P-40 lysis buffer (with the following inhibitors, 100  $\mu$ M phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 100  $\mu$ M sodium vanadate, and 20  $\mu$ g/ml TLCK) with a polytron (Brinkmann Instruments, Westbury, NY), and homogenate supernatants were extracted by centrifugation (14,000  $\times$  g for 20 min at 4  $^{\circ}$ C) (46).

**Cells and Cell Culture**—Primary murine lung fibroblasts were derived from 7–10-week-old C57BL/6 mice and propagated as described previously (47, 48). Briefly, primary cultures of murine lung fibroblasts were established by mechanical disaggregation and enzymatic digestion (collagenase and DNase) of sterilely removed lungs and by cell culture. Fibroblasts were selected by differential adherence to tissue culture plastic and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 20 mM HEPES, and 100 units/ml penicillin/streptomycin/gentamycin. The cell identity of primary cultures was verified by the absence of tight junctions, by production of collagen types I and III, and by typical morphology on phase contrast as described previously (47, 48). Primary adult normal human lung fibroblasts (HL1–1608) were purchased from Cambrex (Walkersville, Maryland), and normal adult human lung fibroblasts (HL2–19LU) were purchased from American Type Culture Collection (Manassas, VA). Human lung fibroblasts were propagated and maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin. The experiments were performed on early passages (passage 2–9) of primary murine and human lung fibroblasts. The Tet-FAK fibroblast-like cells and their parental FAK-null embryonic fibroblasts were kindly provided by Dr. Steven K. Hanks. In the absence of doxycycline in cell culture medium, FAK expression is induced in Tet-FAK cells as described previously (49). In the presence of doxycycline (2  $\mu$ g/ml), FAK expression in Tet-FAK cells is repressed (49). The Tet-FAK cells and their parental FAK-null cells were propagated and maintained in DMEM containing 10% FBS, 4.5 g/liter D-glucose, 0.6 g/liter glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 units/ml penicillin/streptomycin, and 0.25  $\mu$ g/ml amphotericin B as described previously (49).

**Preparation, Amplification, and Purification of the Adenoviral FRNK (Ad-FRNK) Construct**—The HA-tagged FRNK construct was reported previously (50). To efficiently deliver the FRNK cDNA into primary lung fibroblasts, the adenovirally mediated gene delivery system was used. The HA-tagged FRNK cDNA was engineered into a replication-deficient adenovirus vector by using the Adeno-X Expression System 2 according to the manufacturer's manual (Clontech, Mountain View, CA). The Ad-FRNK was rescued and amplified in 293 cells, purified by CsCl gradient centrifugation, and used to infect cells as described previously (46, 51). Adenoviral vectors encoding a green fluorescent protein (Ad-GFP) or a luciferase construct (Ad-Luc) were initially used to optimize the infection conditions on subconfluent (70–80%) cells cultured in serum-containing or serum-free conditions as described previously (46, 51). The cells in serum-free medium (DMEM with 1% BSA) were infected with Ad-FRNK or control vectors (Ad-Luc or Ad-GFP) 24 h prior to TGF- $\beta$ 1 treatment.

**Signal Transduction Experiments without and with MAPK Inhibitors**—Prior to TGF- $\beta$ 1 treatment, fibroblasts were quiescent by growing in DMEM with 0.2% FBS for 24 h, followed in serum-free media (DMEM with 1% BSA) for another 24 h. Quiescent cells were treated with either 10 ng/ml of TGF- $\beta$ 1 or vehicle (Me<sub>2</sub>SO) in serum-free media (DMEM with 1% BSA) for the time indicated in the text. The selective pharmacological inhibitors, PD98059 or U0126 (both are MEK inhibitors), or/and SB203580 (p38 MAPK inhibitor), or U0124 (inactive analog of U0126) were added to culture medium 1 h prior to TGF- $\beta$ 1 treatment.

**Western Blotting**—As described previously (33, 52). The cells were lysed in 1% Nonidet P-40 lysis buffer containing the following inhibitors, 100  $\mu$ M phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 100  $\mu$ M sodium vanadate, and 20  $\mu$ g/ml TLCK. Protein concentration of whole cell or whole lung lysate was determined by a BCA kit (Pierce). Equivalent micrograms (by BCA assay) of whole cell or lung detergent lysates were electrophoresed on a disulfide-reduced 7.5% SDS-PAGE, transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA), probed, or stripped followed by reprobing with indicated antibodies, and developed with ECL system (GE Healthcare). FRNK protein level was determined by Western blot under non-disulfide-reduced conditions following immunoprecipitation of equivalent lysates as described previously (52). The expression of G3PDH protein was used as a loading control. Quantitative analysis (densitometry) of Western blots was performed by calculating the relative density (pixel density) of the immunoreactive bands after acquisition of the blot image (scanning) and analysis with Adobe Photoshop software. For analysis of band intensity, a specific band on the ECL-developed films was subjected to densitometric analysis (Adobe Photoshop). The densitometric readings were pooled and averaged from three independent experiments, and the results were normalized to the total protein level. The background of densitometric reading on the ECL-developed film was subtracted as described previously (33, 53).

**Immunofluorescence Analysis**—Cells cultured on glass coverslips were fixed in 4% buffered paraformaldehyde, permeabilized, and reacted sequentially with primary antibodies, fol-

lowed by secondary antibodies. To detect HA-tagged FRNK expression, the cells were reacted with monoclonal anti-HA IgG (13  $\mu$ g/ml), and followed by goat anti-mouse Alexa 488 IgG (10  $\mu$ g/ml). To study the  $\alpha$ -SMA-incorporated cytoskeletal fibers, the cells were reacted with Cy3-conjugated anti- $\alpha$ -SMA monoclonal antibodies (1:300). Digital fluorescence images were obtained by using a Leica microscope and Simple PCI software (Compix Inc.). The percentage of cells containing highly organized  $\alpha$ -SMA-incorporated fibers were quantified. The results were pooled from eight randomly selected, non-overlapping fields (original magnification, 400 $\times$ ) from a total of three experiments (each performed in triplicate).

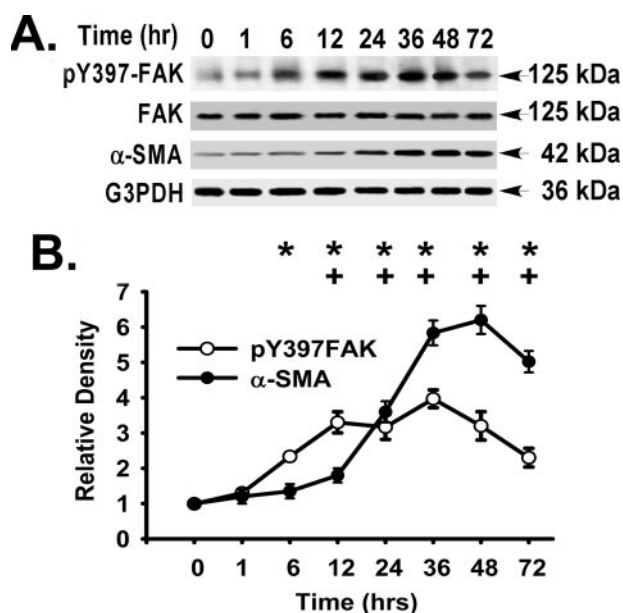
Frozen lung tissue sections (8–10  $\mu$ m) were permeabilized, reacted with Cy3-conjugated monoclonal anti- $\alpha$ -SMA antibody (1:300 dilution), and counterstained with hematoxylin (Sigma-Aldrich). Digital fluorescence images (original magnification, 400 $\times$ ) of stained lung sections were obtained by using a Leica microscope and Simple PCI software (Compix Inc.). The areas containing  $\alpha$ -SMA-positive cells *versus* total parenchymal area on lung sections was quantified by the Simple PCI software (Compix, Inc.). The results were pooled from a total of eight sections/animal (at least 50- $\mu$ m apart). Lesions of comparable histology were used. Areas that represent airways and blood vessels were excluded. Five animals/group (bleomycin or saline) were analyzed.

**Statistical Analysis**—The data were analyzed using the *t* test analysis (Sigma Plot, SPSS Inc.) or one-way analysis of variance (SigmaStat, SPSS Inc.) as indicated and are expressed as the means  $\pm$  S.D. *p* values <0.05 are considered statistically significant. All of the experiments were repeated at least three times.

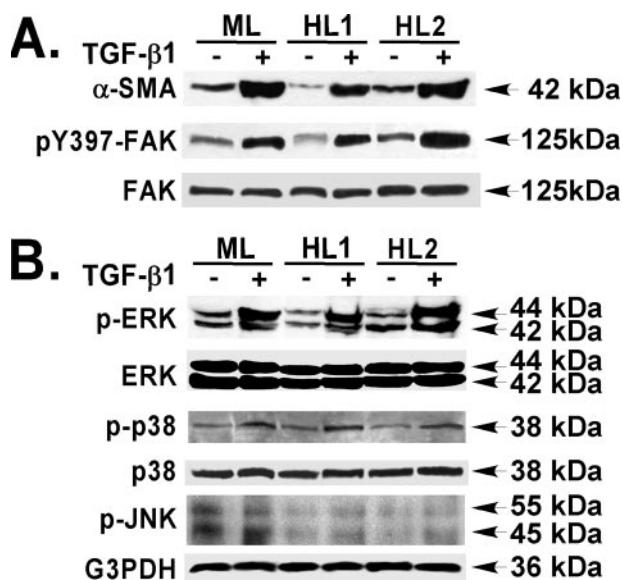
## RESULTS

**TGF- $\beta$ 1 Induces  $\alpha$ -SMA Expression and the Activation of FAK, ERK, and p38 MAPK in Primary Murine and Human Lung Fibroblasts**—To examine the role of FAK in TGF- $\beta$ 1-induced  $\alpha$ -SMA expression (a hallmark of the myfibroblast phenotype) (1), we first compared the time courses of FAK activation (FAK tyrosine 397 phosphorylation) and  $\alpha$ -SMA expression in primary murine lung fibroblasts in response to TGF- $\beta$ 1 stimulation (Fig. 1). FAK activation was persistently elevated from 6 to 72 h, and  $\alpha$ -SMA expression was persistently elevated from 12 to 72 h (Fig. 1). This effect of TGF- $\beta$ 1 on  $\alpha$ -SMA expression and FAK activation was confirmed in additional primary murine and human lung fibroblasts (at 36 h) (Fig. 2A).

Because the MAPK pathway has been shown to exhibit cross-talk with that of TGF- $\beta$  signaling pathway, we measured the activation of MAPK in response to TGF- $\beta$ 1. ERK activation was induced ( $\sim$ 4-fold), and p38 activation was induced ( $\sim$ 3-fold) at the time of peak of FAK activation and  $\alpha$ -SMA expression (at 36 h) (Fig. 2B). In contrast, TGF- $\beta$ 1 had minimal effect on JNK activation (at 36 h) (Fig. 2B). The activation of both ERK and p38 MAPK in response to TGF- $\beta$ 1 were sustained (6–48 h) (data not shown), as shown in other cell types (42, 54, 55). Our data demonstrate that TGF- $\beta$ 1 can induce  $\alpha$ -SMA expression, enhance FAK activation, and induce MAPK (ERK and p38) activation in primary murine and human lung fibroblasts.

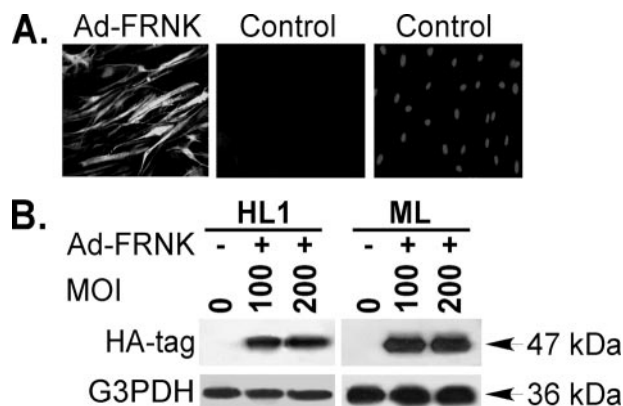


**FIGURE 1. Time course of TGF- $\beta$ 1-induced FAK activation and  $\alpha$ -SMA expression.** Subconfluent (70–80%) primary murine lung fibroblasts were treated with TGF- $\beta$ 1 (10 ng/ml, 37 °C) in serum-free medium (DMEM, 1% BSA) for the indicated times. Equivalent amount of whole cell detergent lysates were Western blotted with the indicated antibodies as described under “Experimental Procedures.” *A*, representative time course of FAK activation (pY397) and  $\alpha$ -SMA expression. *B*, densitometry of FAK activation (pY397-FAK normalized to total FAK protein) and  $\alpha$ -SMA expression (normalized to G3PDH) from *A*. FAK activation (○);  $\alpha$ -SMA expression (●). The data are presented as the means  $\pm$  S.D. ( $n = 3$ ). At the indicated times, + represents  $p < 0.05$  for TGF- $\beta$ 1-induced  $\alpha$ -SMA expression compared with control (without TGF- $\beta$ 1), and \* represents  $p < 0.05$  for TGF- $\beta$ 1-induced FAK activation compared with control (without TGF- $\beta$ 1) by analysis of variance.



**FIGURE 2. TGF- $\beta$ 1 induces  $\alpha$ -SMA expression, FAK activation, and MAPK activation in primary murine and human lung fibroblasts.** Subconfluent primary murine (ML) and human (HL1 and HL2) lung fibroblasts were treated without or with TGF- $\beta$ 1 (10 ng/ml, 36 h) in serum-free medium (DMEM, 1% BSA) as in Fig. 1. Equivalent amounts of whole cell detergent lysates were Western blotted with the indicated antibodies. *A*, FAK activation (pY397) and  $\alpha$ -SMA expression. *B*, ERK, p38, and JNK MAPK activation.

*FRNK Abrogates TGF- $\beta$ 1-induced Incorporation of  $\alpha$ -SMA into Cytoskeletal Fibers and  $\alpha$ -SMA Expression*—FRNK expression inhibits cell migration and proliferation in other cell types

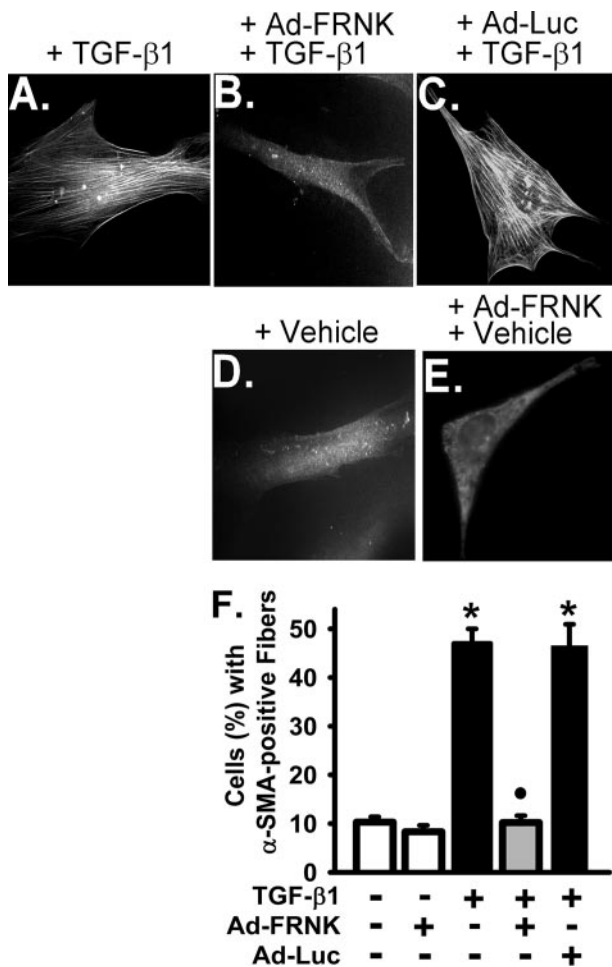


**FIGURE 3. Validation of adenovirus-mediated FRNK expression in lung fibroblasts.** Subconfluent primary murine (ML) or human (HL1) lung fibroblasts were infected with the adenoviral HA-tagged FRNK vector (Ad-FRNK) at a MOI of 100 (*A*) or the indicated MOI (*B*) as described under “Experimental Procedures.” *A*, immunofluorescence analysis of HA-tag for FRNK expression in primary murine lung fibroblasts at 36 h without or with Ad-FRNK infection. *Left panel*, fibroblasts with Ad-FRNK infection and stained with anti-HA antibody. *Middle panel*, fibroblasts with vehicle only stained with anti-HA antibody or *Right panel*, counterstained with Hoechst (nuclear stain). The original magnification was 100 $\times$ . *B*, fibroblasts were lysed at 36 h after Ad-FRNK infection. Equivalent amounts of whole cell detergent lysates were Western blotted with the indicated antibodies. FRNK expression in infected cells was determined by FRNK HA tag expression.

(25), but its effects on myofibroblast differentiation have not been examined. To determine the effect of FRNK on TGF- $\beta$ 1-induced  $\alpha$ -SMA expression, we first validated that the adenoviral HA-tagged FRNK construct (Ad-FRNK) can achieve highly efficient FRNK expression in primary murine and human lung fibroblasts. The efficiency of Ad-FRNK infection was confirmed by immunofluorescent staining directed toward the FRNK HA-tag (Fig. 3*A*). At a multiplicity of infection (MOI) of 100, greater than 95% of the primary murine and human lung fibroblasts were infected by Ad-FRNK, with no increase in cell toxicity (data not shown). The capacity of Ad-FRNK to generate FRNK protein was also confirmed by Western blot by probing for the HA tag in whole cell detergent lysates from primary murine and human lung fibroblasts (Fig. 3*B*).

TGF- $\beta$ 1 induced incorporation of  $\alpha$ -SMA into highly organized cytoskeletal fibers, a phenotypic hallmark of myofibroblast differentiation (1, 56), in primary murine lung fibroblasts (Fig. 4*A*). FRNK expression, as mediated by Ad-FRNK, completely blocked TGF- $\beta$ 1-induced incorporation of  $\alpha$ -SMA into cytoskeletal fibers in these cells (Fig. 4*B*). Control transfections using Ad-Luc had no effect on TGF- $\beta$ 1-induced incorporation of  $\alpha$ -SMA into cytoskeletal fibers (Fig. 4*C*), and FRNK expression mediated by Ad-FRNK had no effect on the basal (vehicle only) level of  $\alpha$ -SMA incorporation into cytoskeletal fibers (Fig. 4*E*). FRNK expression also abrogated TGF- $\beta$ 1-induced  $\alpha$ -SMA expression (by  $\sim$ 85%) (Fig. 5*A*). Control transfection with Ad-Luc had no effect  $\alpha$ -SMA protein expression (Fig. 5*A*). In summary, FRNK expression abrogates TGF- $\beta$ 1-induced myofibroblast differentiation by blocking the effects of TGF- $\beta$ 1 on  $\alpha$ -SMA expression (Fig. 5) and on the formation of  $\alpha$ -SMA-containing cytoskeletal fibers (Fig. 4).

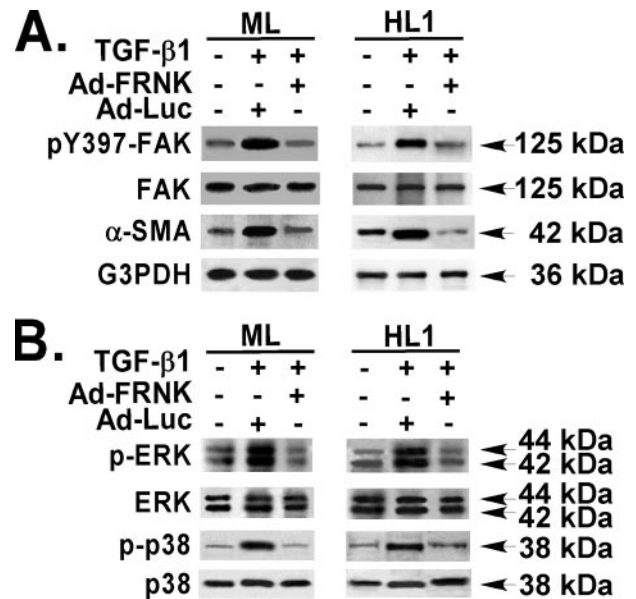
*FRNK Abrogates TGF- $\beta$ 1-induced Activation of FAK, ERK, and p38 MAPK*—Because FAK mediates myofibroblast differentiation, and MAPK can act as a signaling intermediate of both



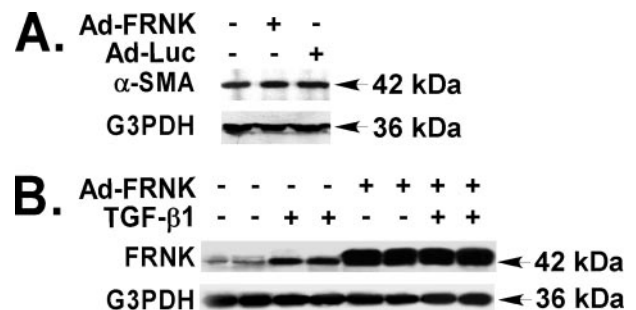
**FIGURE 4. FRNK expression inhibits formation of  $\alpha$ -SMA-containing cytoskeletal fibers in response to TGF- $\beta$ 1 in primary lung fibroblasts.** Subconfluent primary murine lung fibroblasts were infected with Ad-FRNK or control Ad-Luc (adenoviral luciferase construct) at 100 MOI, treated with TGF- $\beta$ 1 (10 ng/ml, 36 h) in serum-free medium (DMEM, 1% BSA), and stained with Cy3-conjugated mouse anti- $\alpha$ -SMA IgG as described under "Experimental Procedures." *A*, immunofluorescence analysis of  $\alpha$ -SMA-containing fibers in primary murine lung fibroblasts with Cy3-conjugated mouse anti- $\alpha$ -SMA IgG (original magnification, 400 $\times$ ). *B*, quantification of the percentage of cells with highly organized  $\alpha$ -SMA-containing fibers as described under "Experimental Procedures." *F*, open bars represent vehicle-treated cells. Filled black bars represent TGF- $\beta$ 1-treated cells. The gray bar represents cells infected with Ad-FRNK and treated with TGF- $\beta$ 1. The data are presented as the means  $\pm$  S.D. ( $n = 3$ , each performed in triplicate). \* represents  $p < 0.01$  for TGF- $\beta$ 1-treated cells compared with cells treated with vehicle only.  $\bullet$  represents  $p < 0.01$  for TGF- $\beta$ 1-treated cells compared with cells infected with Ad-FRNK and treated with TGF- $\beta$ 1.

FAK and TGF- $\beta$ 1 signals, we tested the effect of Ad-FRNK on FAK and MAPK activation in response to TGF- $\beta$ 1. FRNK expression abrogated TGF- $\beta$ 1-induced FAK activation (by  $\sim$ 95%), as measured by the proportion of the Tyr(P)<sup>397</sup> form of FAK relative to total FAK (Fig. 5A). Control transfection with Ad-Luc had no effect on FAK activation (Fig. 5A) in these primary lung fibroblasts. Similarly, FRNK expression completely blocked TGF- $\beta$ 1-induced ERK activation (by  $>$ 95%) and p38 activation (by  $\sim$ 90%) (Fig. 5B). These data demonstrate that FRNK expression abrogates TGF- $\beta$ 1-induced FAK, ERK, and p38 activation.

One consideration is the possibility that adenovirally mediated FRNK expression would result in massive overexpression



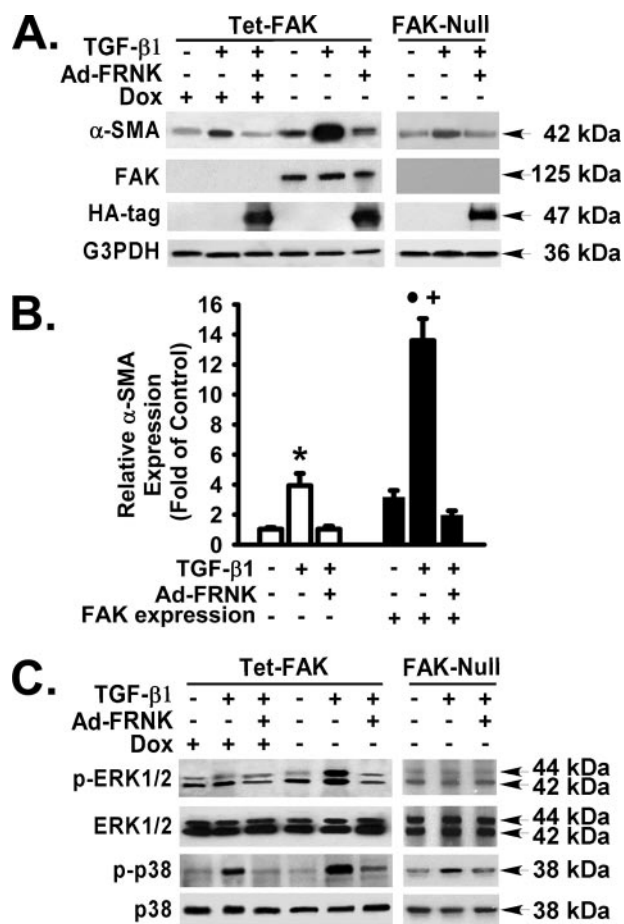
**FIGURE 5. FRNK expression abrogates TGF- $\beta$ 1-induced  $\alpha$ -SMA expression and the activation of FAK, ERK, and p38.** Quiescent primary murine (ML) and human (HL1) lung fibroblasts were infected with Ad-FRNK or control vector (Ad-Luc) (both at 100 MOI), treated with TGF- $\beta$ 1 (10 ng/ml, 36 h) in serum-free medium (DMEM, 1% BSA) and detergent-lysed. *A*, equivalent amount of whole cell lysates were Western blotted with the indicated antibodies as described under "Experimental Procedures." *B*, equivalent amounts of whole cell detergent lysates were also Western blotted for ERK and p38 with the indicated antibodies.



**FIGURE 6. FRNK protein level is increased in primary lung fibroblasts in response to TGF- $\beta$ 1.** *A*, quiescent primary murine lung fibroblasts infected with Ad-FRNK or control Ad-Luc (both at 100 MOI) were detergent-lysed. Equivalent amount of whole cell lysates was used to determine the  $\alpha$ -SMA expression by Western blot as described under "Experimental Procedures." *B*, quiescent primary murine lung fibroblasts infected with or without Ad-FRNK (at 100 MOI) were treated with TGF- $\beta$ 1 (10 ng/ml, 72 h) or vehicle in serum-free medium (DMEM, 1% BSA), and detergent-lysed. Equivalent amounts of whole cell detergent lysates were used to determine the expression of FRNK by Western blot.

of FRNK leading to the unmasking of nonphysiological actions. However, TGF- $\beta$ 1 itself induced FRNK protein expression by 3.7-fold (Fig. 6B), and adenovirally driven FRNK protein levels were only 5.1-fold greater than that detected after TGF- $\beta$ 1 stimulation (Fig. 6B).

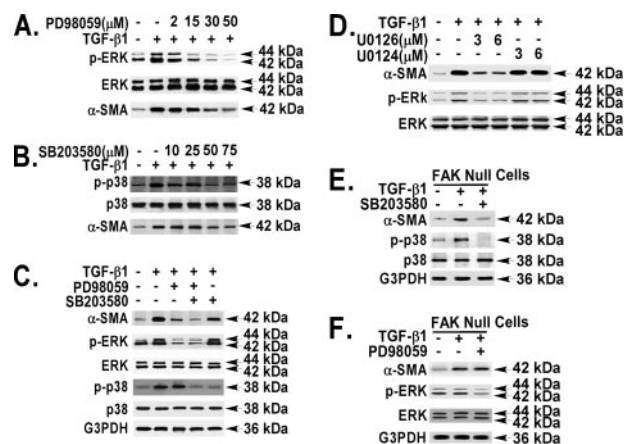
**FRNK Expression Abrogates TGF- $\beta$ 1-induced  $\alpha$ -SMA Expression in the Presence or Absence of FAK**—FRNK is thought to inhibit FAK-mediated signaling through its effects on recruitment of signaling proteins that bind to FAK. To examine whether the inhibitory effect of FRNK on TGF- $\beta$ 1-induced  $\alpha$ -SMA expression is totally dependent on blocking FAK-mediated signaling, the effect of FRNK expression on  $\alpha$ -SMA expression was examined in fibroblasts from the same parental



**FIGURE 7. FRNK expression abrogates TGF-β1-induced α-SMA expression in the presence or absence of FAK.** Tet-FAK fibroblasts were treated with doxycycline (Dox) to repress FAK expression or allowed to express FAK in the absence of doxycycline. Tet-FAK cells (with or without doxycycline) and their parental FAK-null fibroblasts were infected with or without Ad-FRNK and then treated with or without TGF-β1 (10 ng/ml, 36 h) in serum-free medium (DMEM, 1% BSA). *A* and *C*, equivalent amount of whole cell detergent lysates were Western blotted with the indicated antibodies. Representative results are shown. The FRNK HA tag is shown at 47 kDa. *B*, densitometry of α-SMA expression (normalized to G3PDH and relative to FAK-deficient cells without TGF-β1) as described under "Experimental Procedures." α-SMA expression for FAK-deficient cells were pooled from results obtained in Tet-FAK cells (with doxycycline) and FAK-null fibroblasts. *Open bars* denote the results from FAK-deficient cells. *Filled black bars* denote the results from FAK-expressing cells. The data are presented as the means ± S.D. (*n* = 3). \* represents *p* < 0.05 for TGF-β1-treated FAK-deficient cells compared with the same group of cells infected with Ad-FRNK or treated with vehicle only. ● represents *p* < 0.01 for TGF-β1-treated FAK-expressing cells compared with the same group of cells infected with Ad-FRNK or treated with vehicle only. + represents *p* < 0.01 for TGF-β1-treated FAK-expressing cells compared with TGF-β1-treated FAK-deficient cells.

line with regulatable FAK expression. This approach minimizes the effect of inherent differences in α-SMA expression seen among different cell types and lineages (57).

TGF-β1 was capable of inducing α-SMA expression in cells that express FAK, concordant with our results in primary cells (Fig. 7, *A* and *B*). Surprisingly, TGF-β1 was also capable of inducing α-SMA in FAK-deficient fibroblasts, albeit to a submaximal level (13.6 ± 2.5-fold versus 3.9 ± 1.4-fold in the FAK-deficient fibroblasts, *p* < 0.01) (Fig. 7*B*, *fifth bar versus second bar*). This partial FAK dependence was also noted under basal conditions, where FAK-deficient cells had a 3-fold lower level of α-SMA expression (Fig. 7*B*,



**FIGURE 8. ERK and p38 are differentially involved in TGF-β1-induced α-SMA expression in the presence or absence of FAK.** Pharmacologic inhibitor (PD98059, SB203580, or U0126) was added to quiescent primary murine lung fibroblasts (*A–D*) or FAK-null fibroblasts (*E* and *F*) 1 h before TGF-β1 treatment (10 ng/ml, 36 h). The cells were detergent-lysed. Equivalent amounts of whole cell lysates were Western blotted with the indicated antibodies. *A* and *B*, fibroblasts were incubated without or with the indicated concentrations of inhibitors (PD98059 or SB203580). *C–F*, fibroblasts were treated without or with TGF-β1 (10 ng/ml) in the absence or presence of PD98059 (30 μM), SB203580 (50 μM), or U0126 and U0124 at indicated concentrations.

*fourth bar versus first bar*), when compared with FAK-expressing cells. These data demonstrate that FAK has an independent impact on α-SMA induction by TGF-β1. Nonetheless, the FAK signaling pathway is required for maximal α-SMA expression by TGF-β1. Furthermore, these data demonstrate for the first time that FRNK expression can abrogate TGF-β1-induced α-SMA expression in the presence or absence of FAK.

**ERK and p38 MAPK Differentially Mediate TGF-β1-induced α-SMA Expression in a FAK-dependent Manner**—Because we have shown that FRNK expression will block TGF-β-initiated MAPK activation and α-SMA expression, we sought to determine whether the effects of FRNK are a consequence of inhibiting FAK. Both ERK and p38 MAPK were activated in response to TGF-β1, in fibroblasts that express FAK (Fig. 7*C*; also see Fig. 2*B*). In contrast, only p38 was activated in response to TGF-β1, in FAK-deficient fibroblasts (Fig. 7*C*).

To delineate the importance of ERK and p38 MAPKs to the TGF-β1 response in α-SMA expression and its dependence on FAK, specific MAPK pharmacological inhibitors were used in cells that express FAK or lack FAK. As expected, the ERK inhibitor (PD98059) and the p38 inhibitor (SB203580) respectively, blocked TGF-β1-induced ERK and p38 MAPK activation in a selective and dose-dependent manner (Fig. 8, *A* and *B*). Inhibition of both ERK and p38 was required to completely abrogate the TGF-β1-induced α-SMA expression in fibroblasts that express FAK (> 95% by densitometry) (Fig. 8*C*, *fourth lane versus second lane*). ERK was the predominant MAPK (PD98059 blocked by ~80%), whereas p38 played a minor role (SB203580 blocked by ~15%) in the TGF-β1-induced α-SMA expression in FAK-expressing cells (Fig. 8*C*). These findings were confirmed by using a second ERK inhibitor (77% blockage using U0126) (Fig. 8*D*), whereas the inactive control analog, U0124, had no effect (Fig. 8*D*).

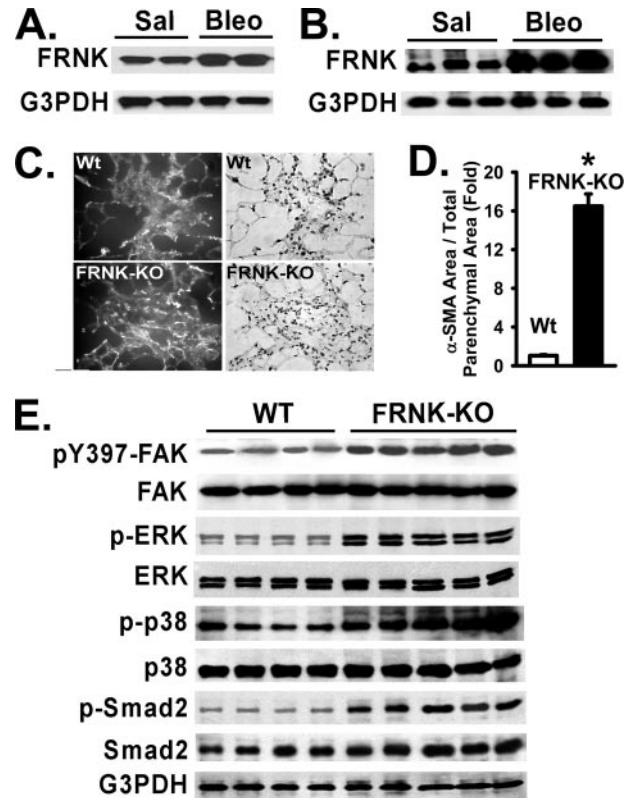
In contrast to our finding in the FAK-expressing fibroblasts, p38 MAPK was solely responsible for the  $\alpha$ -SMA expression in response to TGF- $\beta$ 1 in FAK-deficient cells. This is supported by our observation that the p38 inhibitor SB203580 blocked the  $\alpha$ -SMA expression by  $\sim$ 92% in the FAK-deficient cells (Fig. 8E), whereas the inhibition of ERK had no effect (Fig. 8F). These data demonstrate that the major MAPK utilized in the  $\alpha$ -SMA expression pathway is ERK in FAK-expressing cells and is p38 in FAK-deficient cells (Fig. 8).

**FRNK Inhibits TGF- $\beta$ 1-induced MAPK Activation and  $\alpha$ -SMA Expression in the Absence of FAK**—Because we show that FRNK expression inhibits both MAPK activation and  $\alpha$ -SMA expression in the presence of FAK, we tested the effect of FRNK on these pathways in the absence of FAK. As expected, both ERK and p38 MAPK activation in response to TGF- $\beta$ 1 were blocked by FRNK, in fibroblasts that express FAK (Fig. 7C; also see Fig. 5B). Surprisingly, MAPK (p38) activation and  $\alpha$ -SMA expression in response to TGF- $\beta$ 1 was also blocked by FRNK expression in FAK-deficient fibroblasts (Fig. 7, A and C). ERK was not activated in response to TGF- $\beta$ 1 in FAK-deficient cells (Fig. 7C). These data demonstrate that FRNK expression abrogates TGF- $\beta$ 1-induced  $\alpha$ -SMA expression and MAPK activation in the presence or absence of FAK (Figs. 5 and 7).

We have demonstrated 1) MAPK kinases mediate  $\alpha$ -SMA expression in response to TGF- $\beta$ 1, and 2) ERK is the major downstream signaling intermediate in the pathway from TGF- $\beta$ 1 to  $\alpha$ -SMA in cells that express FAK, whereas 3) p38 is the major downstream signaling intermediate in the pathway from TGF- $\beta$ 1 to  $\alpha$ -SMA in cells that lack FAK, and 4) FRNK expression will inhibit the  $\alpha$ -SMA expression and MAPK activation in both FAK-expressing FAK-deficient fibroblasts. Therefore, we sought to determine whether FRNK inhibits myfibroblast differentiation *in vivo* during the pulmonary fibrotic process.

**FRNK Mediates FAK and MAPK Activation and Myfibroblast Differentiation during Lung Fibrosis *in Vivo***—To determine the role of FRNK in myfibroblast differentiation *in vivo* during experimentally induced pulmonary fibrosis (bleomycin) (3, 5, 20, 45, 58–60), we first measured FRNK protein expression in normal and fibrotic lung tissue, and in primary fibroblast isolates from these lungs. FRNK protein levels were increased  $\sim$ 3.2-fold in fibroblasts isolated from bleomycin-injured lungs, when compared with fibroblasts isolated from saline control lungs (Fig. 9A). Concordantly, FRNK protein was increased  $\sim$ 4.7-fold in whole lung lysates from bleomycin-injured mice, as compared with that from saline controls (Fig. 9B). As a consequence of the loss of FRNK, FAK activation was increased 3-fold in bleomycin-treated FRNK knock-out mice, as compared with wild type (Fig. 9E). Unchallenged mice have similar levels of FAK activity (data not shown).

The activation of the signaling pathways involved in myfibroblast differentiation during bleomycin-induced pulmonary fibrosis was tested in wild type and FRNK knock-out mice. Both ERK and p38 MAPK activation was increased in whole lung lysates from bleomycin-injured FRNK knock-out mice, when compared with bleomycin-injured wild type controls (Fig. 9E). As evidence for TGF- $\beta$  signaling, Smad2



**FIGURE 9. FRNK expression is increased during lung injury, and myfibroblast mass is enhanced in lungs from bleomycin-challenged FRNK knock-out mice.** Lungs were harvested from bleomycin-challenged mice (Bleo) or control saline-treated mice (Sal, 18 days after bleomycin challenge) as described under "Experimental Procedures." *A*, primary lung fibroblasts were isolated from bleomycin-challenged (Bleo) or control saline-treated (Sal) mice and detergent-lysed. Equivalent amounts of whole cell lysate were Western blotted with the indicated antibodies. *B*, equivalent amount of whole lung lysate from bleomycin-challenged or control saline-treated mice were Western blotted with the indicated antibodies. *C*,  $\alpha$ -SMA-positive cells on lung sections obtained from bleomycin-challenged FRNK knock-out (FRNK-KO) and bleomycin-challenged wild type (Wt) mice were analyzed by immunofluorescence staining for  $\alpha$ -SMA as described under "Experimental Procedures." Original magnification, 400 $\times$ . Representative fluorescent images (Top left and bottom left) and bright field (Top right and bottom right) images from wild type mice (top panels) and FRNK-KO mice (bottom panels) are shown. *D*, the total area containing  $\alpha$ -SMA-positive cells in lungs from FRNK-KO and wild type mice shown in *C* was quantitatively determined as described under "Experimental Procedures." The data are presented as the means  $\pm$  S.D. ( $n = 5$ /per group). \* represents  $p < 0.001$  for FRNK-KO mice compared with wild type controls. *E*, equivalent amounts of whole lung lysate from bleomycin-challenged FRNK-KO and bleomycin-challenged wild type (WT) mice were Western blotted with the indicated antibodies.

phosphorylation was also examined and shown to be increased in whole lung lysates from bleomycin-injured FRNK knock-out mice, when compared with that in bleomycin-injured wild type controls (Fig. 9E).

Myfibroblast differentiation in wild type and FRNK knock-out mice was measured by immunofluorescence staining of lung tissue with  $\alpha$ -SMA and semi-quantitative morphometry. The myfibroblast cell area ( $\alpha$ -SMA-positive cell area/total parenchymal area) was strikingly increased  $16.5 \pm 2.8$ -fold ( $p < 0.01$ ) in bleomycin-treated lungs obtained from FRNK knock-out mice, as compared with wild type mice (Fig. 9, C and D). If one focuses on the fibroproliferative lesions, the myfibroblast cell area was increased 3.2-fold in FRNK knock-out mice, as compared with wild type mice (data not shown). The myfibro-

## FRNK Inhibits Myfibroblast Differentiation

blast cell area in unchallenged lung parenchyma of both FRNK knock-out and wild type murine controls was negligible and similar (data not shown). Taken together, these data suggest for the first time that FRNK acts as a brake for myfibroblast differentiation *in vitro* and *in vivo*.

### DISCUSSION

Our studies show that FRNK expression mediated through adenovirus can abrogate TGF- $\beta$ 1-induced  $\alpha$ -SMA expression and modulate MAPK activation in cells that either express FAK or lack FAK. Furthermore, endogenous FRNK expression is increased in fibroblasts in response to TGF- $\beta$  stimulation *in vitro* and in response to the fibrotic agent bleomycin *in vivo*. The *in vivo* physiological relevance of FRNK is demonstrable through the consequences of the loss of FRNK *in vivo*. We show that FRNK-deficient mice have an enhancement of myfibroblast differentiation and MAPK activation *in vivo* in the TGF- $\beta$ -dependent, fibrotic, bleomycin-injured animal model. Taken together, our data demonstrate that FRNK is a novel, negative regulator of myfibroblast differentiation and functions to limit myfibroblast generation *in vivo*, in response to tissue injury.

Despite the importance of FRNK in inhibiting integrin-mediated cell migration and proliferation through the inhibition of FAK, little progress has been made in the understanding of the mechanism by which FRNK expression is regulated. Nonetheless, FRNK expression is up-regulated in smooth muscle cells *in vivo* after balloon-induced vascular injury (37, 39). Furthermore, FRNK expression is increased in vascular smooth muscle cells plated on perlecan, with consequent impaired FAK activation and cell proliferation (61). Our study demonstrates for the first time that FRNK expression is up-regulated in response to a specific profibrotic cytokine, TGF- $\beta$ 1. Furthermore, FRNK expression is up-regulated in fibrotic lung tissue, and in primary isolates of fibroblasts from fibrotic lungs. Because TGF- $\beta$ 1 is considered a key mediator of both fibrosis and myfibroblast differentiation in the bleomycin model, we speculate that TGF- $\beta$ 1 is the physiological inducer of FRNK expression *in vivo* during the fibrotic response (1, 3, 4, 62).

It is well characterized that FRNK inhibits integrin-mediated cell migration through blocking FAK activation and FAK-mediated signaling (25, 37, 39, 40, 50). FRNK comprises the C-terminal region of FAK, including the proline-rich (SH3-binding domains) and focal adhesion targeting domains, but lacks the signaling kinase domain and integrin/cytokine-binding FERM domain. FRNK localizes to focal adhesions through its focal adhesion targeting domain and is thought to inhibit cell migration either through the competitive replacement of FAK in focal adhesions/contacts or by the competitive recruitment of critical signaling proteins away from FAK (25, 40, 63). Our demonstration of a role for FRNK in cell trans-differentiation is entirely novel. Our studies extend the described inhibitory effect of FRNK on integrin-mediated cell migration and proliferation to myfibroblast differentiation *in vitro* and *in vivo*. Furthermore, we show that the inhibitory effect of FRNK on TGF- $\beta$ 1-induced

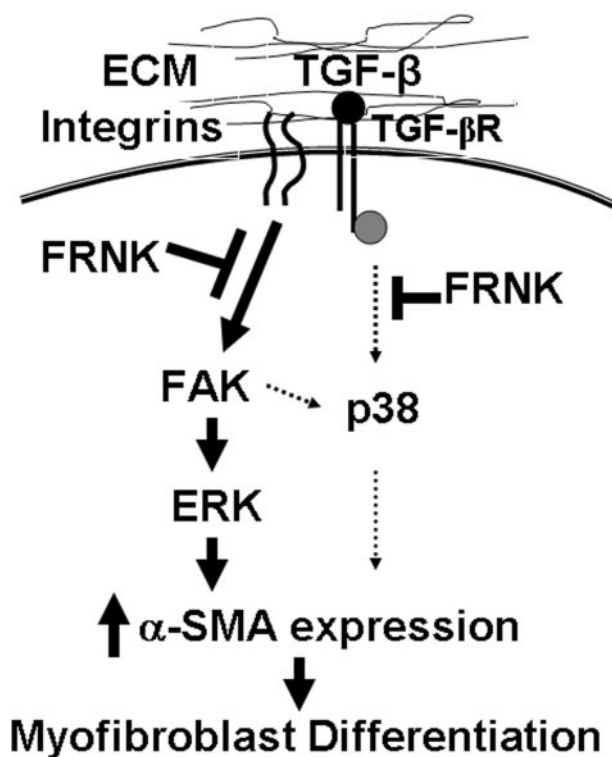


FIGURE 10. Hypothetical mechanism whereby FRNK inhibits TGF- $\beta$ -induced  $\alpha$ -SMA expression in the presence and absence of FAK. TGF- $\beta$ -induced  $\alpha$ -SMA expression and myfibroblast differentiation requires the involvement of multiple signaling initiated through the integrins, extracellular matrix (ECM) environment, and TGF- $\beta$  receptors (TGF- $\beta$ R). Recent evidence demonstrates that certain integrins, in cooperation with the cytoskeleton, are capable of activating latent TGF- $\beta$  through binding to latency-associated protein (21, 26, 68). TGF- $\beta$  induces  $\alpha$ -SMA expression through two pathways; the major pathway through ERK MAPK is FAK-dependent, and the minor pathway through p38 MAPK is FAK-independent. FRNK can inhibit TGF- $\beta$ -induced  $\alpha$ -SMA expression through inhibition of FAK activation and inhibition of the FAK/ERK-mediated pathway. FRNK also can inhibit the pathway of TGF- $\beta$ -induced  $\alpha$ -SMA expression mediated by p38, potentially through inhibition of critical signaling proteins upstream of p38 (*i.e.* Src kinase).

$\alpha$ -SMA expression is largely, but not solely, through inhibition of FAK-mediated signaling.

Other investigators have shown that expression of dominant negative FAK inhibits TGF- $\beta$ 1-induced  $\alpha$ -SMA expression (34). Because complete deletion of FAK may have a different mechanism of action compared with the Tyr<sup>397</sup>  $\rightarrow$  Phe mutant FAK (34), we further tested the dependence of the  $\alpha$ -SMA response to TGF- $\beta$ , on the presence of FAK in both FAK-null and FAK-expressing cells. Surprisingly, we found that TGF- $\beta$ 1 can induce  $\alpha$ -SMA expression in the absence of FAK, albeit to a much lower level. The presence of FAK facilitates basal  $\alpha$ -SMA expression and significantly augments TGF- $\beta$ 1-induced  $\alpha$ -SMA expression. This is supported by two lines of evidence. First, we found that the basal (no TGF- $\beta$ )  $\alpha$ -SMA expression level is 3-fold greater in cells with FAK compared with those without FAK. Second, we found that the maximal response of  $\alpha$ -SMA to TGF- $\beta$ 1 is 3.6-fold greater in cells with FAK compared with cells without FAK (Fig. 7B, fifth bar versus second bar). These data demonstrate that the maximal level of  $\alpha$ -SMA expression can be achieved through both FAK-independent and FAK-dependent signaling pathways (Fig. 10).



The enhanced  $\alpha$ -SMA expression in the presence of FAK may be due to the role of FAK as an important mediator in integrin-mediated signaling, focal adhesion turnover, cytoskeleton remodeling, and mechanosensing processes (3, 4, 25, 32, 64–67). TGF- $\beta$ 1 alone is not sufficient to induce optimal  $\alpha$ -SMA expression (1, 23). Integrin-mediated signaling and expression of EDA fibronectin (alternative splice form of fibronectin), as well as a rigid matrix environment, are required for TGF- $\beta$ 1-induced  $\alpha$ -SMA expression and formation of mature focal adhesion complexes (1, 23). The necessity of integrin-mediated signaling is further supported by the observation that TGF- $\beta$ 1 is not able to induce  $\alpha$ -SMA expression in suspended human lung fibroblasts, although the response of Smad2 to TGF- $\beta$ 1 is intact in these cells (34). Recent evidence demonstrates that certain integrins, in cooperation with cytoskeletons, are capable of activating latent TGF- $\beta$  through binding to latency-associated protein (21, 26, 68). Myofibroblast differentiation is associated with the formation of mature focal adhesion complexes and the incorporation of  $\alpha$ -SMA into cytoskeletal fibers, and it also depends on the presence of a rigid matrix environment (1, 23). FAK regulates integrin-mediated focal adhesion and cytoskeletal remodeling and is required for mechanosensing of a rigid extracellular environment (25, 32, 67). Therefore, we would anticipate that expression of FRNK would inhibit myofibroblast differentiation, through inhibition of FAK activation. In fact, we show that TGF- $\beta$ 1-induced FAK activation and  $\alpha$ -SMA expression are reduced in FRNK-expressing fibroblasts.

It is known that MAPK is required for TGF- $\beta$ -induced expression of extracellular matrix proteins (41–43, 69). Although selective utilization of a MAPK has been reported for TGF- $\beta$ -induced gene expression, for example, type I collagen expression (41–43, 54, 69–71), our observation that differential utilization of both ERK and p38 MAPK upon TGF- $\beta$ 1 stimulation in the same cell and in a FAK-dependent manner is novel. The differential role of ERK and p38 in TGF- $\beta$ 1-induced  $\alpha$ -SMA expression was substantiated in this study by using complementary pharmacological inhibitors. We found that a complete abrogation of TGF- $\beta$ 1-induced  $\alpha$ -SMA expression requires blockade of both ERK and p38 activation and that ERK is the major regulatory MAPK for TGF- $\beta$ 1-induced  $\alpha$ -SMA expression in the presence of FAK. In contrast, we found that p38 is the major regulatory MAPK for TGF- $\beta$ 1-induced  $\alpha$ -SMA expression in the absence of FAK but retains a minor regulatory role in FAK-expressing cells. Although the p38 MAPK pathway exists in wild type cells, the p38 pathway does not compensate for the loss of ERK activation in FAK-deficient cells, thereby resulting in a net reduction in the effect of TGF- $\beta$  on  $\alpha$ -SMA expression when ERK activation is blocked. Nonetheless, the presence of FAK enhances p38 activation in response to TGF- $\beta$ 1, as compared with that in FAK-deficient cells (Figs. 7C and 10).

We show that expression of FRNK, or deletion of FAK (as in FAK-null cells), completely abrogates ERK activation in response to TGF- $\beta$ . This is consistent with previous findings of other investigators demonstrating that ERK activation induced by epidermal growth factor or serum is significantly impaired in FAK-deficient cells (72, 73). The impaired ERK activation they

noted in suspended cells was rescued by expression of a constitutively active FAK, and expression of dominant negative FAK mutant blocked ERK activation, supporting the concept that FAK is an obligate upstream mediator of ERK activation (29, 33, 73). FAK has been shown to mediate ERK activation through multiple pathways. For example, binding of Grb2 to FAK (Y925) leads to subsequent activation of the Ras, Raf, MEK, and ERK signaling pathway (25). Alternatively, we have shown that the negative regulator of Ras activation, p120RasGAP, can be sequestered away through binding to active FAK, thereby augmenting Ras activity and downstream ERK activation (29, 33).

Our study also shows the novel finding that FRNK expression inhibits TGF- $\beta$ 1-induced p38 MAPK activation in the absence of FAK. This is the first demonstration of a FAK-independent physiological function for FRNK. In subsequent studies we will address the exact mechanism by which FRNK inhibits p38 activation. However, the FAK C-terminal domain (FRNK), contains two proline-rich regions, which have been shown to bind SH3 domain-containing proteins (*i.e.* p130Cas and GRAF) (25, 66). Src kinase contains an SH3 domain (74, 75), and Src has been shown to mediate TGF- $\beta$ -induced p38 activation through phosphorylation of TGF- $\beta$ R2 independent of FAK and Smad (76–78). We therefore speculate that FRNK may bind to the SH3 domain of Src through its proline-rich region, thereby recruiting Src away from the TGF- $\beta$ R2. This would have the effect of blunting Src-mediated phosphorylation of the TGF- $\beta$ R2, and the subsequent downstream activation of p38 MAPK. Through this pathway, FRNK can inhibit p38 MAPK activation in a Smad- and FAK-independent manner.

It is only through a complete understanding of the extensive cross-talk between integrin and TGF- $\beta$ 1 signaling pathways that mediate myofibroblast differentiation that we will be able to design efficient strategies to mitigate organ fibrosis and excess scar formation. Our study demonstrates that FAK mediates ERK activation necessary for optimal  $\alpha$ -SMA expression in response to TGF- $\beta$ 1, and we show that a FAK-independent rescue pathway exists that requires p38 MAPK (Fig. 10). Interestingly, FRNK can block both pathways. This is physiologically relevant as endogenous FRNK is up-regulated in response to TGF- $\beta$ 1 *in vitro* and during the fibroproliferative process *in vivo*. Our observations in FRNK-deficient mice indicate that FRNK acts as a physiologic brake on the formation of myofibroblasts during the fibroproliferative process *in vivo*. Thus, to the extent that myofibroblasts mediate excess tissue fibrosis, FRNK may function to limit pathological tissue fibrosis in response to vascular and/or lung or other organ injury.

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