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Downregulation of FAK-Related Non-Kinase Mediates the Migratory Phenotype of Human Fibrotic Lung Fibroblasts

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Abstract

Fibroblast migration plays an important role in the normal wound healing process; however, dysregulated cell migration may contribute to the progressive formation of fibrotic lesions in the diseased condition. To examine the role of focal-adhesion-kinase (FAK)-related non-kinase (FRNK) in regulation of fibrotic lung fibroblast migration, we examined cell migration, FRNK expression, and activation of focal adhesion kinase (FAK) and Rho GTPase (Rho and Rac) in primary lung fibroblasts derived from both idiopathic pulmonary fibrosis (IPF) patients and normal human controls. Fibrotic (IPF) lung fibroblasts have increased cell migration when compared to control human lung fibroblasts. FRNK expression is significantly reduced in IPF lung fibroblasts, while activation of FAK, Rho and Rac are increased in IPF lung fibroblasts. Endogenous FRNK expression is inversely correlated with FAK activation and cell migration rate in IPF lung fibroblasts. Forced exogenous FRNK expression abrogates the increased cell migration, and blocked the activation of FAK and Rho GTPase (Rho and Rac), in IPF lung fibroblasts. These data for the first time provide evidence that downregulation of endogenous FRNK plays a role in promoting cell migration through FAK and Rho GTPase in fibrotic IPF lung fibroblasts.

Keywords

focal adhesion kinase; FAK-related non-kinase; fibroblast; migration; fibrosis; lung

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Introduction

Fibroblasts are identified in normal wound healing locations and in fibrotic lesions. Small aggregates of fibroblasts found in fibrotic lungs of idiopathic pulmonary fibrosis (IPF) patients are known as the “leading edge” of the unstoppable fibrotic response in IPF [1]. IPF is a chronic lung disorder resulting in respiratory insufficiency due to progressive lung fibrosis [1]. Previous data demonstrate that lung fibroblasts from IPF patients have increased cell migration across basement membranes when compared to normal human lung fibroblasts [2]. Integrins and extracellular matrix proteins play a critical role in the enhanced cell migration observed in IPF patient-derived lung fibroblasts [2]. These observations suggest that increased lung fibroblast migration into the wounded or remodeling area may be one of the contributing factors to the exuberant fibrotic response seen in the lungs of IPF patients.

Focal adhesion kinase (FAK) plays an important role in integrin-mediated cell migration [3–5]. FAK-deficient fibroblasts show significantly decreased cell migration [6], and re-expression of FAK in FAK-deficient cells restores the cell migration [7,8]. FAK can be activated by integrin engagement with extracellular matrix proteins, and this results in the phosphorylation of tyrosine 397 (Y397) of FAK [3,4,7]. The phosphorylation of Y397 of FAK plays an important role in FAK-mediated cell migration [3,4,7,9]. Expression of the mutated Y397F FAK effectively inhibits FAK-mediated cell migration [3,10,11]. Increased FAK expression and increased Y397 phosphorylation of FAK have been found in many tumor tissues, and in tumor cell lines that show an increased cell migration rate [12,13]. Increased tumor cell migration is involved in tumor progression and invasion [14].

FAK-related non-kinase (FRNK) is an independently expressed cytoplasmic protein that has the identical protein sequence to the C-terminal region of FAK [3,15]. Overexpression of exogenous FRNK inhibits FAK activation (determined by Y397 phosphorylation of FAK) initiated by integrin receptor engagement with extracellular matrix proteins or by growth factors [3,16,17]. Overexpression of FRNK inhibits FAK-mediated cell migration and proliferation [3,8,18]. FRNK contains the focal adhesion target (FAT) domain as does FAK [3], and published data show that the FAT domain directs FRNK to focal adhesion complexes through binding to integrin-associated proteins (such as paxillin) [14,19]. So far, very little is known regarding the role of endogenous FRNK during normal homeostasis and/or in disease. The expression of FRNK and activation of FAK have yet to be determined in fibrotic lung fibroblasts.

In this study, endogenous FRNK expression was examined in primary normal human and fibrotic IPF lung fibroblasts, and the findings were correlated with the migratory phenotype of normal and IPF lung fibroblasts. The results demonstrate that FRNK expression is significantly decreased in IPF lung fibroblasts. Endogenous FRNK expression has an inverse relationship with cell migration and FAK activation in IPF lung fibroblasts. FAK activation is positively correlated with cell migration in IPF lung fibroblasts. Forced exogenous FRNK expression abrogated the increased cell migration, and the increased FAK and Rho GTPase (Rho and Rac) activation, in IPF lung fibroblasts. These data for the first time provide evidence that endogenous FRNK plays a role in determining cell migration in diseased (IPF) lung fibroblasts through FAK and RhoGTPase, and suggest that downregulation of FRNK may play a role in the pathogenesis of IPF.

Material and Methods

Reagents and Antibodies

Platelet-derived growth factor (PDGF-BB) was obtained from R&D Systems (Minneapolis, MN). The following purified polyclonal antibodies were purchased: anti-phospho-FAK

[pY397] (Biosource, Camarillo, CA), anti-FAK (recognizes both FAK C-terminal and FRNK, Upstate Biotechnology, Lake Placid, NY). Purified Alexa-Fluor-488 goat anti-mouse IgG was purchased from Molecular Probes (Carlsbad, CA). The following purified monoclonal antibodies (mAb) were purchased: anti-Hemagglutinin (HA) epitope tag, anti-FAK and FRNK (Santa Cruz Biotechnology), and anti-glyceraldehyde 3-phosphate dehydrogenase (G3PDH) (Research Diagnostics, Flanders, NJ). Fibronectin, Collagen (Type I), and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cells and Cell Culture

De-identified primary adult normal human lung fibroblasts (NHL) and IPF patient-derived lung fibroblasts (IPF) were purchased from the following vendors: Cambrex (Walkersville, Maryland) and the American Type Culture Collection (ATCC) (Manassas, VA), or derived from explanted lungs at the University of Michigan and the University of Alabama at Birmingham as described previously [2,20,21]. Informed consent was obtained from all subjects at the University of Michigan and the University of Alabama at Birmingham. All cell lines and tissues were de-identified by suppliers and the studies have obtained approvals from the institutional review board (IRB). Lung fibroblasts were maintained and propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin/streptomycin/gentamycin as described previously [2]. Experiments were performed on early passages (passage 3–10) of primary normal human and IPF patient-derived human lung fibroblasts.

Cell Migration Assays

The 2-D wound closure monolayer/scratch motility assay was performed as described previously [22]. Briefly, fibroblasts were harvested with buffered EDTA, resuspended in serum-free Dulbecco's modified Eagle's medium with 1% BSA, and plated into 24-well format tissue culture plates (1×10^5 cells/well). At 24 hour, the monolayer was scratched, digital pictures were taken immediately after wounding/scratching, and again at the end of the assay. The digital pictures were used to calculate the areas of scratch without cells immediately after scratching and the remaining areas without cells at the end of the assay. The wound area covered by cell migration after scratching was equal to the difference between the two areas above, and was normalized to that in normal human lung fibroblast line 19LU (purchased from ATCC) or as indicated. Mitomycin C was added to inhibit cell proliferation after PDGF-BB treatment. The haptotactic cell migration assay was performed in two-well Boyden-type chambers as described previously [23]. Briefly, human lung fibroblasts (4×10^4 cells) in serum-free DMEM media with 1% BSA were plated onto 8 μ m filters coated on the bottom surface with collagen type I (10 μ g/ml) or fibronectin (10 μ g/ml), and allowed to migrate at 37°C with 5% CO₂ for 6 hours. Fibroblast cells on the upper filter surface were removed, and the cells (migrated to) on the lower filter surface were fixed, stained, and counted. Conditions were assayed in replicas of three or four, repeated two to four times, and the data analyzed and presented as the mean $\pm$ SE.

Cell Attachment Assay

The cell attachment assay was performed as described previously [24]. Briefly, human lung fibroblasts were harvested with buffered EDTA, washed, and resuspended in serum free Dulbecco's modified Eagle's medium with 1% BSA. Cells (15,000/well) were plated onto collagen (10 μ g/ml), fibronectin (10 μ g/ml), or ovalbumin (10 μ g/ml)-coated wells, allowed to attach at 37°C for 60 minutes, the wells washed twice with PBS; the attached cells fixed, stained with crystal violet, dried, solubilized in acetic acid, and the absorbance for each well determined in an ELISA reader. Attachment to ovalbumin was subtracted as background.

Preparation, Purification, and Infection of the Adenoviral-FRNK (Ad-FRNK) and Adenoviral-GFP (Ad-GFP) Constructs

The Hemagglutinin (HA)-tagged FRNK construct was reported previously [25]. To efficiently deliver the FRNK cDNA into primary normal and IPF patient-derived human lung fibroblasts, the adenoviral-mediated gene delivery system was used as described previously [26]. Adenoviral vectors encoding a green fluorescent protein (Ad-GFP) was initially used to optimize the infection conditions in primary human normal lung fibroblasts in serum-containing or serum-free conditions [26]. Fibroblasts were infected with Ad-FRNK or control Ad-GFP 24 hours prior to the described experiments. Green fluorescent protein (GFP) expression mediated through the same adenoviral vector (Ad-GFP) was used to optimize the transfection conditions and also served as a control for overexpression. Greater than 90% of cells were GFP-positive, and the percentage was similar in both the normal and IPF lung fibroblasts after cells were treated with Ad-GFP at 100 MOI for 36–48 hours (data not shown).

Immunofluorescence Analysis for HA-FRNK Expression

Fibroblasts cultured on glass-coverslips were fixed in 4% buffered paraformaldehyde, permeabilized, and reacted with monoclonal anti-HA IgG (13 µg/ml), followed by goat anti-mouse Alexa-488 IgG (10 µg/ml) as described previously [23]. Digital fluorescent images were obtained by using a Leica microscope and Simple PCI software (Compix Inc.) to calculate the percentage of HA-positive fibroblasts after Ad-FRNK infection in both primary normal human and IPF patient-derived lung fibroblasts.

Western Blotting

Western blotting assays were performed as described previously [5,22]. Briefly, cells were lysed in 1% NP-40 lysis buffer containing the following inhibitors, PMSF, Aprotinin, Leupeptin, Sodium Vanadate, and TLCK. The protein concentration of the whole cell or whole lung lysate was determined by BCA kit (Pierce, Rockford, IL). Equivalent micrograms of whole cell detergent lysates were electrophoresed on a disulfide-reduced 12% SDS PAGE, transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA), probed and stripped followed by re-probing with indicated antibodies, and developed with the enhanced chemiluminescent (ECL) system (Pharmacia Biotech, Piscataway, NJ). FRNK protein level was determined by Western blot on non-disulfide-reduced condition following immunoprecipitation of equivalent lysates as described previously [22]. The expression of G3PDH protein was used as a loading control. For densitometric analysis of band intensity, a specific band on the ECL-developed film was subjected to densitometric analysis (Adobe Photoshop) [26]. The densitometric readings were pooled and averaged from three independent experiments. The background of densitometric reading on the ECL-developed film was subtracted.

Rac and Rho Activation Assays

Rac and Rho activation were determined by the level of active Rac and active Rho forms (GTP-bound form) in cells. Serum-starved lung fibroblasts were lysed as per the instructions in the kits from Upstate Cell Signaling Solutions (Temecula, CA), and equivalent micrograms of lysate reacted with (for Rac activation) p21-activated kinase-1 binding domain coupled to agarose or (for Rho activation) Rhotekin Rho-binding domain coupled to agarose, and immunoprecipitates subjected to 10% SDS-PAGE, transferred to Immobilon, and Western blotted with anti-Rac IgG or anti-Rho IgG as described previously [23].

Statistical Analysis

Data were analyzed using the unpaired or paired *t*-test analysis (for comparisons between two groups) (Sigma Plot, SPSS Inc.), and expressed as means ± SE. Experiments were performed

two to four times with duplicates. Linear regression was performed by using Sigma Plot (SPSS Inc.). A p value of < 0.05 was considered statistically significant.

Results

Primary IPF lung fibroblasts demonstrate enhanced cell migration when compared to primary normal human lung fibroblasts

The monolayer wound healing closure assay was used to examine whether there is a difference in cell migration phenotype between fibrotic lung fibroblasts derived from IPF patients (IPF lung fibroblasts) and normal human lung fibroblasts. Monolayers of serum starved human lung fibroblasts (both normal and IPF) were wounded, and cell migration was first examined under basal (serum free media, SFM) conditions. Fibroblast movement into the wounded area was tracked, and the relative wound area covered within 24 hours analyzed (Fig. 1A). Under basal conditions, IPF lung fibroblasts demonstrated a significantly increased cell migration into the wounded area (1.3-fold greater, $n = 10$, $p < 0.01$) when compared to normal human lung fibroblasts (NHL) (Fig. 1B).

To examine cell migration in response to platelet derived growth factor BB (PDGF-BB), monolayers of serum-starved human lung fibroblasts (both normal and IPF) were wounded, and cell migration was tracked in the absence and presence of PDGF-BB (4 ng/ml) for 24 hours at 37°C. In response to PDGF-BB treatment, cell migration was significantly increased in both normal (2.5-fold) and IPF (3.4-fold) lung fibroblasts when compared to that in normal lung fibroblasts at serum-free condition (Fig. 1B). The results also indicate that there is a difference in PDGF-BB-stimulated cell migration between IPF and normal lung fibroblasts. In response to PDGF-BB treatment, IPF lung fibroblasts migrate faster (about 36% faster) than normal lung fibroblasts (Fig. 1B). Cell attachment was also determined as described in the Materials and Methods. The findings indicate that cell attachment on either collagen (10 μ g/ml), or fibronectin (10 μ g/ml) at 37°C (5% CO₂, 60 minutes) is similar between the normal and IPF lung fibroblasts (data not shown). The above data demonstrate that IPF lung fibroblasts have an enhanced cell migration phenotype when compared to normal lung fibroblasts in both basal and PDGF-BB-stimulated conditions.

Endogenous FRNK protein level is decreased, and is inversely correlated with cell migration rate in primary IPF lung fibroblasts

FRNK overexpression has been used as a tool to inhibit integrin-mediated cell migration [3, 16,17]. However, little is known regarding the function of endogenous FRNK with regard to cell migration. To investigate whether FRNK contributes to the enhanced cell migration observed in IPF lung fibroblasts (as shown in Fig. 1), FRNK expression was examined in both IPF and normal human (NHL) lung fibroblasts. FRNK protein level was significantly decreased in IPF lung fibroblasts (mean of 42%, ranging from 11% to 112%, $n = 10$, $p < 0.05$) when compared to that in normal lung fibroblasts (Figs. 2A and 2B). The level of downregulation of FRNK expression varies among IPF lung fibroblast lines. The largest decrease of FRNK expression was seen in IPF lung fibroblast lines with a faster cell migration rate (Fig. 2A, top panel).

Linear regression analysis indicates that FRNK protein level is inversely correlated with cell migration rate in IPF lung fibroblasts (Fig. 2C, $n = 10$, $p < 0.01$). In contrast, there is no relationship between FRNK protein level and cell migration rate in normal human lung fibroblasts ($n = 6$, data not shown). It is well known that FRNK overexpression inhibits FAK activation; however, the effect of downregulation of FRNK expression on FAK activation has not been studied before. As FRNK expression is significantly decreased in IPF lung fibroblasts, we examined FAK expression and activation in both IPF and normal human lung fibroblasts.

Similar total FAK protein levels were found in IPF and normal lung fibroblasts (Fig. 2E). However, FAK activation (determined by Y397 phosphorylation of FAK) was significantly increased in IPF lung fibroblasts (by 73%, $n = 10$, $p < 0.05$) when compared to that in normal lung fibroblasts (Fig. 2D).

To determine whether the changes in FRNK expression and cell migration correlate with FAK activation, linear regression analysis was performed. FRNK expression is inversely correlated with FAK activation in IPF lung fibroblasts (Fig. 2F, $n = 10$, $p < 0.01$). FAK activation is positively/directly correlated with cell migration in IPF lung fibroblasts (Fig. 2G, $n = 10$, $p < 0.01$). There was no similar correlation found between FRNK expression and FAK activation, or between FAK activation and cell migration, in normal human lung fibroblasts (data not shown). The above results indicate that IPF lung fibroblasts have an imbalanced FRNK/FAK axis (decreased FRNK expression and increased FAK activation), and this imbalance of the FRNK/FAK axis is strongly correlated with the increased cell migration observed in IPF lung fibroblasts.

Exogenous FRNK expression abrogates the enhanced FAK activation and cell migration in primary IPF lung fibroblasts

To further understand the mechanistic links among FRNK expression, FAK activation, and cell migration in lung fibroblasts, the effect of exogenous FRNK expression on FAK activation and cell migration was examined in both IPF and normal human lung fibroblasts. Expression of the hemagglutinin (HA)-tagged FRNK was confirmed by immunofluorescent staining (Fig. 3A) and Western blot (Fig. 3B) in both IPF and normal human lung fibroblasts. At a multiplicity of infection (MOI) of 100, over 95% of lung fibroblasts express the HA-tagged FRNK (Fig. 3A). FRNK expression mediated by Ad-FRNK abrogated the increased FAK activation (as detected by decreased Y397 phosphorylation of FAK) in IPF lung fibroblasts ($n = 2$), and also slightly decreased FAK activation in normal lung fibroblasts (NHL, $n = 2$) (Figs. 3B and 3C). Control green fluorescent protein (GFP) expression mediated by the adenoviral vector (Ad-GFP) had no effect on the increased FAK activation in IPF lung fibroblasts (Figs. 3B and 3C), and had no effect on the basal FAK activation level in normal lung fibroblasts (data not shown).

The effect of exogenous FRNK expression on cell migration was then examined by monolayer wound healing closure assay (as shown in Fig. 1). FRNK expression mediated by Ad-FRNK abrogated the increased basal (in serum-free condition) and PDGF-BB-stimulated cell migration in IPF lung fibroblasts ($n = 9$) (Fig. 4B). In normal human lung fibroblasts ($n = 5$), FRNK expression mediated by Ad-FRNK completely blocked the PDGF-BB-stimulated cell migration (Fig. 4A), and slightly decreased cell migration in the basal condition (Fig. 4A). Exogenous GFP expression mediated by Ad-GFP (as a control) had no effect on cell migration in both normal human and IPF lung fibroblasts in both basal (data not shown) and PDGF-BB-stimulated conditions (Figs. 4A and 4B, respectively).

Haptotactic cell migration is the directed movement of cells toward an insoluble gradient of extracellular matrix (ECM) proteins, such as collagen or fibronectin. IPF lung fibroblasts ($n = 9$) demonstrated a significantly increased cell migration toward both fibronectin and collagen when compared to normal human lung fibroblasts ($n = 5$) (Figs. 5A and 5B, second bar versus first bar), further supporting that IPF lung fibroblasts have a migratory phenotype. FRNK expression mediated by Ad-FRNK abrogated the increased cell migration toward fibronectin and collagen in IPF lung fibroblasts (Figs. 5A and 5B). Furthermore, FRNK expression mediated by Ad-FRNK also significantly decreased cell migration toward both fibronectin and collagen in normal human lung fibroblasts (Figs. 5A and 5B). Exogenous GFP expression mediated by Ad-GFP had no effect on cell migration toward both fibronectin and collagen in normal human or in IPF lung fibroblasts (Figs. 5A and 5B).

Exogenous FRNK expression abrogates the enhanced Rac and Rho activation in primary IPF lung fibroblasts

Rho family GTPases are signaling intermediates of cell migration, and they regulate cell migration through modulating actin polymerization and cytoskeletal reorganization [27–29]. Whether they play a role in the increased IPF lung fibroblast migration is not yet known. Activation of two major Rho family GTPases (Rac and Rho) was examined in normal human (NHL, n = 5, individual lines) lung fibroblasts, and in IPF (IPF, n = 5, individual lines) lung fibroblasts that have an increased cell migration (as shown in Fig. 1). Rac and Rho activation were determined by the level of active Rac and active Rho forms (GTP-bound forms) in lung fibroblasts, as described previously [23]. Rac activation was significantly increased (about 3-fold) in IPF lung fibroblasts when compared to that in normal lung fibroblasts (Figs. 6A and 6B). Rho activation was also increased (about 1.8-fold) in IPF lung fibroblasts when compared to that in normal lung fibroblasts (Figs. 6A and 6C).

We then examined the effect of exogenous FRNK expression on Rac and Rho activation in these normal human and IPF lung fibroblasts. FRNK expression mediated by Ad-FRNK abrogated the increased Rac activation in IPF lung fibroblasts, and decreased Rac activation in normal human lung fibroblasts (Fig. 6B). FRNK expression also abrogated the increased Rho activation in IPF lung fibroblasts (Fig. 6C). NHL (n=5) and IPF (n=5) lung fibroblasts without adenoviral construct infection (representative image from one cell line each group was shown in Fig. 6A), or with Ad-GFP (expressing GFP) served as controls. Exogenous GFP expression mediated by Ad-GFP had no effect on the Rac and Rho activation in both normal and IPF lung fibroblasts (Fig. 6).

Discussion

Our study demonstrates that IPF lung fibroblasts have decreased FRNK expression, increased FAK and RhoGTPase (Rho and Rac) activation, and increased cell migration when compared to normal human lung fibroblasts. FRNK expression is inversely correlated with FAK activation, and is inversely correlated with cell migration rate in IPF lung fibroblasts. FAK activation is directly correlated with cell migration rate in IPF lung fibroblasts. Forced exogenous FRNK expression abrogates the increased cell migration, and inhibits the increased activation of FAK, Rac and Rho in IPF lung fibroblasts. Our data for the first time show that downregulation of endogenous FRNK promotes fibrotic lung fibroblast migration through FAK and Rho GTPases.

Our results confirm and extend the previous findings that IPF lung fibroblasts have an increased migratory phenotype when compared to normal human lung fibroblasts [2]. Changes in lung fibroblast migration are thought to contribute to the pathogenesis of IPF [30,31]. Our data demonstrate that IPF fibrotic lung fibroblasts as a group have increased cell migration when compared to normal human lung fibroblasts. Increased FAK activation is known to promote cell migration through regulation of focal adhesion turnover and cytoskeleton re-organization [3,32]. The role of FAK in IPF lung fibroblast migration has not been studied previously. Our results show that FAK activation is increased in migratory IPF lung fibroblasts, and FAK activation is directly correlated with cell migration in IPF lung fibroblasts (Fig. 1 and Fig. 2). Small Rho GTPases, such as Rac and Rho, also regulate cell migration through modulating actin polymerization and lamellipodia formation [27–29]. Activation of Rac and Rho is increased in migratory IPF lung fibroblasts (Fig. 6), and this is not known previously. The evidence in the literature suggests that Rac and Rho are downstream intermediates of FAK-dependent cell migration [27–29]. Our findings are consistent with a mechanism whereby increased FAK activation enhances cell migration through promoting Rac and Rho activity in IPF lung fibroblasts. Inhibition of FAK activation (by forced exogenous FRNK expression) abrogates the increased cell migration in IPF lung fibroblasts. These results indicate that FAK

activation is important for IPF lung fibroblast migration, and this is consistent with the role of FAK in cell migration seen in other cell types. Nonetheless, our results support that inhibition of FAK by pharmacological reagents can be one novel therapeutic intervention to limit the fibroblast migration during progressive fibrotic tissue remodeling.

The physiologic role of endogenous FRNK in cell migration is rarely known. Our data for the first time describe that downregulation of FRNK expression is inversely correlated with increased FAK activation and cell migration in a diseased condition (IPF fibrotic lung fibroblasts). Restoring FRNK protein level by forced exogenous FRNK expression inhibits the increased cell migration and FAK activation in IPF lung fibroblasts, supporting that the decreased FRNK expression contributes to the increased cell migration and FAK activation in IPF lung fibroblasts. FRNK expression level varies among the different types of cell and tissue, and FRNK expression level is relatively high in the lung [33]. The role of endogenous FRNK during normal homeostasis and/or in disease in vivo has yet to be determined. Our data indicate that endogenous FRNK may function as a negative regulator of lung fibroblast migration during normal wound healing, and downregulation of endogenous FRNK expression may lead to exuberant lung fibroblast migration in fibrotic lungs.

How FRNK inhibits cell migration is not completely understood. It is hypothesized that FRNK inhibits cell migration by inhibition of FAK activation, either through the competitive replacement of FAK in focal adhesions and/or by the competitive recruitment of critical signaling proteins away from FAK [3,8,17,18]. Our data show that FRNK inhibits both FAK activation and cell migration in fibrotic IPF lung fibroblasts, and this is consistent with findings from other cell types [3]. Our results further show that FRNK inhibits RhoGTPase activation in IPF lung fibroblasts. Forced FRNK expression abrogates the increased Rac and Rho activation in IPF lung fibroblasts. It is possible that downregulation of FRNK contributes to the increased Rac and Rho activation, and restoring FRNK expression inhibits Rac and Rho activation through inhibition of FAK activation in IPF lung fibroblasts. The exact mechanism by which FRNK inhibits Rac and Rho is not addressed by this study. Whether FRNK inhibits Rac and Rho activation through the FAK-dependent, FAK-independent, or both pathways will be studied in future work.

In order to design targeted therapies for progressive fibrotic diseases (such as IPF), it is important to understand the signaling pathways contributing to the progressive development of fibrotic lesions. Our study demonstrates that IPF lung fibroblasts have decreased FRNK expression, and the decrease of FRNK expression is inversely correlated with the increase of cell migration rate and FAK activation in IPF lung fibroblasts. FAK activation is directly correlated with cell migration rate in IPF lung fibroblasts. Inhibition of FAK and RhoGTPase activation by restoring FRNK expression abrogates the increased cell migration in IPF lung fibroblasts. Together, our findings indicate that a dysregulated FRNK/FAK axis (decreased FRNK expression and increased FAK activation) contributes to the migratory phenotype in IPF lung fibroblasts. As fibroblast migration is considered as one contributing factor in the development of progressive fibrotic lesions, we speculate that a dysregulated FRNK/FAK axis could be one mechanism for the inexorable fibrotic response in IPF lungs. Pharmacological reagents targeting the dysregulated FRNK/FAK axis may be used to limit the progressive fibrotic remodeling in IPF lungs.

Abbreviations

IPF	idiopathic pulmonary fibrosis
FAK	focal adhesion kinase
FRNK	FAK-related non-kinase

TGF- β 1	transforming growth factor beta-1
Ad-FRNC	adenoviral FRNC construct
GFP	green fluorescent protein
PDGF-BB	platelet derived growth factor BB
MOI	multiplicity of infection
IP	immunoprecipitate
GEF	guanine nucleotide exchange factor
G3PDH	glyceraldehyde 3-phosphate dehydrogenase
HA	Hemagglutinin epitope tag

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Fig. 1. IPF lung fibroblasts demonstrate enhanced cell migration under basal (serum-free) and PDGF-BB-stimulated conditions

(A): Serum-starved normal human lung fibroblasts were wounded, and the monolayer wound area was monitored for 24 hours at 37°C. Representative digital images at 0-hour and 24-hour are shown. (B): Migration of normal human lung fibroblasts (NHL) and IPF lung fibroblasts (IPF) were examined as in Panel A. NHL and IPF lung fibroblasts were wounded and treated without or with PDGF-BB (4 ng/ml) in serum-free media (SFM) with 1% BSA as in Panel A. Data were pooled from six NHL and ten IPF human lung fibroblast lines, and plotted (mean + SE) as % of wound area covered over 24 hours relative to normal human lung fibroblasts (NHL). The wound area covered over 24 hours in 19LU normal human lung fibroblasts was

defined as 100%. The black bars represent the IPF lung fibroblasts. * represents $p < 0.01$ relative to NHL group in SFM. # represents $p < 0.01$ relative to NHL group treated with PDGF-BB.

Fig. 2. FRNK protein level is decreased and FAK activation is increased in IPF lung fibroblasts
(A): Normal human lung fibroblasts (NHL, $n = 6$), and IPF lung fibroblasts (IPF, $n = 10$) were examined. IPF lung fibroblasts with an increased cell migration rate were presented at the Top-Panel (IPF, faster rate, $n = 6$). IPF lung fibroblasts ($n = 4$) having a similar cell migration rate as NHL were presented in the Bottom-Panel. Endogenous FRNK expression was examined by immunoprecipitation (IP) of equivalent amount of whole cell detergent lysates, and Western blotted with antibody directed toward FRNK. Equivalent amount of whole lysates was also Western blotted with the indicated antibodies. **(B):** Densitometry of FRNK protein level from NHL and IPF lung fibroblasts, and were normalized to G3PDH level. The dot represents each individual line. The data are represented as the percentage of FRNK level relative to that in

19LU normal human lung fibroblasts. The bar represents the average of each group. **(C)**: Linear regression analysis of FRNK protein level versus IPF lung fibroblast migration rate (relative to 19LU as shown in Fig. 1) (n = 10). **(D) and (E)**: Densitometry of FAK activation and total FAK (as shown in *Panel A*), respectively. The data were normalized to G3PDH, and represented as the fold of FAK activation or expression relative to that in 19LU normal human lung fibroblasts. **(F)**: Linear regression analysis of FRNK protein level versus FAK activation in IPF lung fibroblasts (n = 10). **(G)**: Linear regression analysis of FAK activation versus IPF lung fibroblast migration rate (relative to 19LU as shown in Fig. 1) (n = 10).

Fig. 3. Exogenous FRNK expression abrogates increased FAK activation in IPF lung fibroblasts (A): HA-tagged FRNK expression was mediated by adenoviral vector (Ad-FRNK), and confirmed by immunofluorescence staining in normal human (NHL) and IPF (IPF) lung fibroblasts at the indicated multiplicity of infection (MOI). Digital images were obtained (10X), and plotted against the MOI (mean $\pm$ SE). (B): NHL (n = 2) and IPF (n = 2) lung fibroblasts were infected with either Ad-FRNK or control vector encoding the green fluorescent protein (Ad-GFP) at 100 MOI, and lysed after 36 hours. Equivalent amount of whole cell detergent lysates were Western blotted with the indicated antibodies. (C): Densitometry of FAK activation (normalized to total FAK protein) from Panel B. The data are represented as the fold activation of FAK relative to NHL group infected with control Ad-GFP.

Fig. 4. Exogenous FRNK expression mediated by adenoviral vector (Ad-FRNK) abrogates increased cell migration in IPF lung fibroblasts, and inhibits PDGF-BB-stimulated cell migration in both normal human and IPF lung fibroblasts

Normal human lung fibroblasts (NHL, $n = 5$) and IPF lung fibroblasts (IPF, $n = 9$) were infected with Ad-FRNK or control Ad-GFP, and subjected to the monolayer wound healing assay as shown in Fig. 1. (A): wounded NHLs and (B): wounded IPFs were treated without or with PDGF-BB (4 ng/ml) for 24 hours in serum-free media (SFM) with 1 % BSA at 37°C. The wound area was monitored and quantified as in Fig. 1. Data were pooled and plotted (mean $\pm$ SE) as the % of wound area covered over 24 hours relative to NHL in basal (SFM) condition.

* and # represent $p < 0.001$.

Fig. 5. Exogenous FRNK expression mediated by adenoviral vector (Ad-FRNK) inhibits cell migration toward fibronectin and collagen in both normal human and IPF lung fibroblasts Normal human (NHL, $n = 5$) and IPF (IPF, $n = 9$) lung fibroblasts were infected with Ad-FRNK or control Ad-GFP as in Fig. 4. Cells were then plated onto filters coated on the bottom surface with $10 \mu\text{g/ml}$ of fibronectin (shown in *Panel A*:) or $10 \mu\text{g/ml}$ of collagen (shown in *Panel B*:), allowed to migrate for 6 hours (37°C , $5\% \text{ CO}_2$), and haptotactic cell migration was determined as described in the “METHODS”. The data were analyzed and presented as the mean $\pm$ SE. * represents $p < 0.01$.

Fig. 6. Increased Rac and Rho activation were found in IPF lung fibroblasts, and exogenous FRNK expression abrogates increased Rac and Rho activation in IPF lung fibroblasts

(A): Normal human (NHL, $n = 5$) and IPF (IPF, $n = 5$) lung fibroblasts were lysed at 36 hours after Ad-FRNK or control Ad-GFP infection, as in Fig. 3. Rac and Rho activation were examined as described in the "METHODS". Equivalent amount of whole cell detergent lysates were also Western blotted for total Rac and Rho. NHL ($n=5$) and IPF ($n=5$) lung fibroblasts without adenoviral construct infection (No Ad) served as a control, and representative image from one cell line each group was shown. (B) and (C): Densitometry of Rac and Rho activation from Panel A (normalized to total Rac and Rho protein, respectively). The data are represented as the fold change relative to the normal human lung fibroblasts infected with Ad-GFP.