

Correlational analysis of TRAF5-JNK and integrin β 1-FAK signaling in human lung fibroblasts cultured on decellularized fibrotic versus normal lung extracellular matrix

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Abstract: Background: Pulmonary fibrosis involves pathological remodeling of the extracellular matrix (ECM), which actively influences fibroblast behavior. The specific signaling pathways associated with fibrotic ECM-driven fibroblast activation remain incompletely defined. In particular, TNF receptor-associated factor 5 (TRAF5), a signaling adaptor traditionally studied in immune cells, has never been investigated as an ECM-responsive molecule in fibroblasts. **Objectives:** To investigate the effects of decellularized normal (N-ECM) and fibrotic (F-ECM) lung ECM on human lung fibroblast (HLF) metabolic activity and activation phenotype and to quantify associations with TRAF5-JNK and integrin β 1-FAK signaling. **Methods:** Decellularized ECM scaffolds were prepared from normal (n=3) and fibrotic (IPF, n=3) human lung tissues, validated by DNA quantification (<50 ng/mg) and histology. Primary HLFs were cultured on ECM-coated plates (100 μ g/cm²) for 24 hours. Metabolic activity was assessed by CCK-8 (with ECM-only controls); cytotoxicity by LDH release; protein expression by Western blot with densitometric normalization to GAPDH and total protein (p-JNK/JNK, p-FAK/FAK ratios); mRNA by qPCR; and α -SMA localization by immunofluorescence with ImageJ quantification. Pearson correlation analysis was performed to explore relationships between pathway components and activation markers. **Results:** F-ECM significantly increased HLF metabolic activity compared to Control (1.8-fold, 95% CI: 1.5–2.1, P<0.01) without elevating LDH release (P>0.05). ECM-only control wells showed no CCK-8 interference (OD450 < 0.05). Densitometric analysis demonstrated that F-ECM increased TRAF5 protein (2.4-fold, P<0.01), p-JNK/JNK ratio (2.1-fold, P<0.05), integrin β 1 (2.2-fold, P<0.01) and p-FAK/FAK ratio (2.3-fold, P<0.01) compared to Control. The α -SMA protein (3.1-fold, P<0.01) and mRNA (4.2-fold, P<0.01) were also upregulated. TRAF5 and α -SMA protein expression showed a strong positive correlation (r=0.89, 95% CI: 0.71–0.96, P<0.001). Immunofluorescence confirmed increased α -SMA fluorescence intensity (2.8-fold, P<0.01) and spindle-shaped morphological changes in F-ECM-treated cells. **Conclusion:** Decellularized fibrotic lung ECM is associated with increased fibroblast metabolic activity and activation marker expression, correlating with upregulated TRAF5-JNK and integrin β 1-FAK signaling. These correlational findings provide hypothesis-generating evidence for ECM-driven mechanisms in pulmonary fibrosis but require validation through pathway inhibition or gene knockdown studies to establish causality. TRAF5 is identified as a candidate ECM-responsive signaling molecule warranting further mechanistic investigation.

Keywords: Decellularized matrix; Extracellular matrix; Fibroblast activation; Integrin β 1-FAK signaling pathway; Pulmonary fibrosis; TRAF5-JNK signaling pathway

Submitted on 17-04-2026 – Revised on 06-07-2026 – Accepted on 20-07-2026

INTRODUCTION

Pulmonary fibrosis, particularly idiopathic pulmonary fibrosis (IPF), is a progressive and fatal interstitial lung disease characterized by pathological accumulation of the extracellular matrix (ECM), leading to irreversible respiratory failure (Zuo *et al.*, 2025). Despite antifibrotic therapies, median survival remains 3-5 years, reflecting an incomplete understanding of the molecular mechanisms that drive sustained fibrosis (Zhang *et al.*, 2025, Qiu *et al.*, 2025). The ECM is no longer viewed as a passive scaffold but as a dynamic microenvironment that actively regulates cellular behavior (Boaru *et al.*, 2025). During fibrosis progression, the lung ECM

undergoes profound changes in composition, organization and mechanical properties (stiffness increasing from ~5 kPa to >25 kPa), which actively contribute to disease pathogenesis by creating a positive feedback loop: fibrotic ECM promotes fibroblast activation and myofibroblast differentiation, which in turn deposit more fibrotic ECM (Ganzleben and Medoff, 2025).

Previous studies using decellularized ECM scaffolds have demonstrated that fibrotic ECM alone can induce fibroblast activation, characterized by upregulation of alpha-smooth muscle actin (α -SMA), increased proliferation and enhanced contractility (Pot *et al.*, 2023; Mayorca-Guiliani *et al.*, 2025). However, conflicting

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reports exist regarding the specific signaling pathways involved. While integrin β 1-FAK signaling is established as a key mechanotransduction axis, the role of TNF receptor-associated factor (TRAF) family members in non-immune cells remains poorly understood (Katoh, 2025). TRAF5, traditionally studied in immune receptor signaling (e.g., TNFR, CD40), has never been investigated as an ECM-responsive molecule in fibroblast biology (Hikosaka-Kuniishi *et al.*, 2024). Whether TRAF5 participates in ECM-mediated fibroblast activation and how it might relate to integrin-FAK signaling is completely unknown. This represents a critical knowledge gap: while ECM-driven fibroblast activation is well-documented, the specific intracellular signaling pathways that transduce ECM-derived signals, particularly TRAF family members in non-immune cells, remain undefined.

Several inconsistencies in the literature warrant attention. First, while some studies report that integrin β 1-FAK signaling is universally activated by stiff ECM (Chao *et al.*, 2025), others suggest that ECM composition, not stiffness alone, determines signaling outcomes (Wang *et al.*, 2021). Second, the relative importance of chemical versus mechanical ECM cues remains debated; studies using decellularized ECM cannot distinguish these variables, whereas studies using synthetic hydrogels cannot replicate the complexity of native ECM (Antczak *et al.*, 2024). Third, while JNK activation has been linked to fibroblast proliferation in some contexts (Wang *et al.*, 2021), other studies have implicated JNK in apoptosis, suggesting context-dependent effects (Antczak *et al.*, 2024, Khan *et al.*, 2026a). This study addresses these inconsistencies by simultaneously measuring both pathways in a single experimental system.

The central problem addressed by this study is whether decellularized fibrotic lung ECM is associated with altered fibroblast activation markers and concurrent changes in TRAF5-JNK and integrin β 1-FAK signaling. While decellularized ECM models have demonstrated that fibrotic ECM alone can induce fibroblast activation (Ganzleben and Medoff, 2025), the specific intracellular signaling pathways that transduce ECM-derived signals remain incompletely characterized. In particular, TRAF5, a signaling adaptor traditionally studied in immune cells, has never been investigated as an ECM-responsive molecule in fibroblasts. Furthermore, the relationship between TRAF5-JNK and integrin β 1-FAK signaling in the context of ECM-driven fibroblast activation has not been explored.

How might acellular ECM activate TRAF5, a molecule classically involved in cytokine receptor signaling? Three testable hypotheses exist: (1) fibrotic ECM may sequester TNF- α or other TRAF5-activating cytokines from the culture medium; (2) integrin-mediated adhesion may

transcriptionally upregulate TRAF5 via NF- κ B or AP-1; or (3) cellular stress induced by abnormal ECM adhesion could activate TRAF5 via reactive oxygen species pathways. Each hypothesis remains speculative and requires experimental testing.

This is the first study to: (1) quantify TRAF5 expression in response to acellular fibrotic ECM in any cell type; (2) simultaneously measure TRAF5-JNK and integrin β 1-FAK signaling in the same experimental system; and (3) provide correlational evidence linking these pathways to fibroblast activation markers in human lung fibroblasts. Therefore, the specific objectives of this study were to:

- (1) Quantify the effect of fibrotic ECM (F-ECM) versus normal ECM (N-ECM) on HLF metabolic activity using the CCK-8 assay, with ECM-only controls to rule out interference and assess cytotoxicity by LDH release.
- (2) Measure protein expression of TRAF5, p-JNK, total JNK, integrin β 1, p-FAK, total FAK and α -SMA by Western blot with densitometric normalization to GAPDH and calculation of p-JNK/JNK and p-FAK/FAK ratios.
- (3) Quantify mRNA expression of TRAF5, ITGB1 and ACTA2 by qPCR using the $2^{-(\Delta\Delta Ct)}$ method with GAPDH normalization.
- (4) Assess α -SMA localization and cell morphology by immunofluorescence with ImageJ quantification of mean fluorescence intensity normalized to DAPI-positive nuclei.

By addressing these objectives, this study provides foundational, correlational evidence for associations between fibrotic ECM and specific signaling pathways, thereby identifying candidate molecular targets (*TRAF5*, *JNK*, *integrin β 1*, *FAK*) for future mechanistic interrogation. Importantly, this study does not prove causality; definitive determination of whether these pathways mediate ECM-induced fibroblast activation will require targeted inhibitor and gene knockdown experiments. Nonetheless, these hypothesis-generating data address a critical gap in understanding how ECM-derived signals may be transduced in pulmonary fibrosis.

MATERIALS AND METHODS

Cells and reagents

Human lung fibroblasts (HLF) were obtained from a standard cell bank and cultured in DMEM (Gibco, 11965092) medium containing (FBS, Gibco, 26140079) and 1% penicillin-streptomycin (Gibco, 15140122) at 37°C in a 5% CO₂ incubator. Cells were passaged until reaching the logarithmic growth phase and used for subsequent experiments. Decellularized lung ECM was derived from normal and fibrotic lung tissue. After

decellularization, normal ECM (N-ECM) and fibrotic ECM (F-ECM) were obtained and used for cell stimulation experiments following sterile processing. The CCK-8 assay kit and LDH release assay kit were purchased from MCE (USA); TRAF5, p-JNK, integrin β 1, p-FAK, α -SMA and β -actin antibodies were purchased from Proteintect; Jima Biotechnology synthesized qPCR-related reagents and primers; secondary antibodies and DAPI dye used for immunofluorescence staining were purchased from MCE (USA).

Lung tissue pieces (1 cm³) were subjected to decellularization using a modified detergent-enzymatic method. Tissues were washed in distilled water for 24 h, then incubated in 0.1% SDS (sodium dodecyl sulfate) in deionized water with gentle agitation at room temperature for 72 h, with solution changes every 24 h. Tissues were washed in PBS for 24 h, followed by incubation in 1% Triton X-100 in PBS for 48 h at room temperature. After three PBS washes (1 h each), tissues were treated with DNase I (50 U/mL) and RNase A (1 mg/mL) in 10 mM Tris-HCl (pH 7.5) containing 2.5 mM MgCl₂ for 3 h at 37°C. Finally, samples were extensively washed in PBS for 72 h with multiple changes.

The detergent-enzymatic decellularization method was chosen over alternative approaches (e.g., high hydrostatic pressure, supercritical CO₂) because it effectively removes cellular material while preserving ECM architecture, as confirmed by residual DNA <50 ng/mg and retention of collagen architecture on Masson's trichrome staining. This method has been validated by multiple independent groups for lung ECM studies (Mendibil *et al.*, 2020).

Decellularization efficacy was confirmed by: (1) DAPI staining showing absence of visible nuclei; (2) DNA quantification using Quant-iT PicoGreen dsDNA assay (Thermo Fisher), with residual DNA <50 ng/mg dry weight in all samples (normal: 32.6±8.4 ng/mg; fibrotic: 41.2±10.1 ng/mg; both below the accepted threshold of <50 ng/mg); (3) HandE staining showing no visible nuclear material; and (4) Masson's trichrome staining confirming retention of collagen architecture.

Experimental groups and treatments

After seeding, HLF cells were divided into the following three groups. Control group: cultured in standard medium; N-ECM group: treated with decellularized normal lung extracellular matrix; F-ECM group: treated with decellularized fibrotic lung extracellular matrix. Subsequent assays were performed 24 hours after treatment in each group.

CCK-8 assay for cell viability

HLF metabolic activity was assessed using the Cell Counting Kit-8 (CCK-8, MCE, Monmouth Junction, NJ,

USA). Cells were seeded in 96-well plates (5×10³ cells/well, 100 μ L medium) and treated as above. After 24 hours, 10 μ L of CCK-8 solution was added to each well and plates were incubated for 1.5 hours at 37°C. Absorbance at 450 nm (reference 650 nm) was measured using a microplate reader (BioTek Synergy H1, Winooski, VT, USA). To rule out ECM interference with the CCK-8 assay, ECM-only control wells (coated with N-ECM or F-ECM, no cells) were included in all experiments. All ECM-only controls showed absorbance <0.05 OD₄₅₀, confirming no detectable interference. Results are expressed as fold-change relative to the Control group after subtracting background absorbance. Three biological replicates (each with three technical replicates) were performed. The CCK-8 assay was validated for this system by (1) confirming linearity of absorbance versus cell number from 1×10³ to 2×10⁴ cells/well (R²=0.995); (2) ruling out ECM interference through ECM-only control wells (OD₄₅₀<0.05); and (3) confirming that CCK-8 reduction did not vary significantly with ECM coating density (tested at 50, 100 and 200 μ g/cm², coefficient of variation <5%).

LDH release assay

Cell membrane integrity was assessed using the LDH-Glo™ Cytotoxicity Assay (MCE, USA). After 24 hours of treatment, cell culture supernatants were collected and centrifuged at 300×g for 5 minutes. LDH release was measured according to the manufacturer's protocol using a luminometer (BioTek Synergy H1). Maximum LDH release (positive control) was determined by treating cells with 1% Triton X-100 for 10 minutes. Results are expressed as a percentage of maximum LDH release. Three biological replicates (each with three technical replicates) were performed.

Protein expression

Following treatment, cells were washed twice with ice-cold PBS and lysed in RIPA buffer (Beyotime, Shanghai, China, P0013B) supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher, 78442, 1× final concentration). Lysates were sonicated (three 5-second pulses) and centrifuged at 14,000×g for 15 minutes at 4°C. Protein concentration was determined using the BCA assay (Thermo Fisher, 23225). Equal amounts of protein (30 μ g per lane) were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, IPVH00010, 0.45 μ m). Membranes were blocked with 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 hour at room temperature, then incubated with primary antibodies overnight at 4°C (Table 1 for catalog numbers, clones and dilutions). After three TBST washes (10 minutes each), membranes were incubated with HRP-conjugated secondary antibodies (1:5000, Cell Signaling, 7074 for rabbit, 7076 for mouse) for 1 hour at room temperature.

Table 1: Antibodies used in the study.

Antibody	Source	Catalog no.	Clone	Dilution (WB)	Dilution (IF)
TRAF5	Proteintech	66678-1-Ig	Monoclonal (mouse)	1:1000	-
p-JNK (Thr183/Tyr185)	Cell signaling	4668	Rabbit mAb	1:1000	-
JNK	Cell signaling	9252	Rabbit mAb	1:1000	-
Integrin β 1 (ITGB1)	Proteintech	55272-1-AP	Rabbit pAb	1:2000	-
p-FAK (Tyr397)	Cell signaling	3283	Rabbit mAb	1:1000	-
FAK	Cell signaling	3285	Rabbit mAb	1:1000	-
α -SMA	Proteintech	67735-1-Ig	Mouse mAb	1:2000	1:400
GAPDH	Proteintech	60004-1-Ig	Mouse mAb	1:5000	-
β -actin	Proteintech	66009-1-Ig	Mouse mAb	1:5000	-

Signals were visualized using ECL chemiluminescence substrate (Thermo Fisher, 34580) and imaged using a ChemiDoc MP system (Bio-Rad, Hercules, CA, USA). Densitometric analysis was performed using ImageJ software (NIH, version 1.53). p-JNK and p-FAK signals were normalized to total JNK and total FAK, respectively, to calculate p-JNK/JNK and p-FAK/FAK ratios. TRAF5, integrin β 1 and α -SMA were normalized to GAPDH. All Western blots were performed with three biological replicates.

qPCR analysis of gene expression

Total RNA was extracted using TRIzol reagent (Invitrogen, 15596026) according to the manufacturer's protocol. RNA concentration and purity were assessed by Nanodrop (A260/A280 >1.8, A260/A230 >2.0). Reverse transcription was performed using the PrimeScript RT Reagent Kit (Takara, RR037A) with 1 μ g of total RNA. Quantitative PCR was performed using TB Green Premix Ex Taq II (Takara, RR820A) on a CFX96 Real-Time System (Bio-Rad). Cycling conditions were: 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. Melting curve analysis confirmed single-product amplification. Primer sequences were as follows: TRAF5 forward 5'-CAGCCACTACTCCGTGTTCC-3', reverse 5'-TGCTGGATGTCCTGGTAGGT-3'; ITGB1 (integrin β 1) forward 5'-GATGCCATCATGCAAGTGGAG-3', reverse 5'-GGTTGGCAGCGTAGTAATGG-3'; ACTA2 (α -SMA) forward 5'-CCCAGACATCAGGGAGTAATGG-3', reverse 5'-TCTATCGCTCATCCCTGGTTC-3'; GAPDH forward 5'-GGAGCGAGATCCCTCCAAAT-3', reverse 5'-GGCTGTTGTCATACTTCTCATGG-3'. All reactions were performed in triplicate. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method with GAPDH as the endogenous control (Khan *et al.*, 2025). Three biological replicates were performed.

Immunofluorescence staining

HLFs were seeded on sterile glass coverslips (12 mm diameter) placed in 24-well plates at a density of 1×10^4 cells per well. After 24 hours of ECM treatment, cells were washed twice with PBS, fixed in 4% paraformaldehyde for 15 minutes at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 10

minutes and blocked with 5% bovine serum albumin (BSA, Sigma, A7030) in PBS for 1 hour. Coverslips were incubated with mouse anti- α -SMA antibody (Proteintech, 67735-1-Ig, 1:400 dilution in 1% BSA) overnight at 4°C. After three PBS washes (5 minutes each), coverslips were incubated with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (Invitrogen, A11001, 1:500) for 1 hour at room temperature in the dark. Nuclei were counterstained with DAPI (1 μ g/mL, Invitrogen, D1306) for 5 minutes. Coverslips were mounted using ProLong Gold Antifade Mountant (Invitrogen, P36930). Images were captured using a fluorescence microscope (Olympus IX83, Tokyo, Japan) with a 20 $\times$ objective (NA 0.75, total magnification 200 $\times$). For quantification, five random fields per coverslip were captured using identical exposure settings (500 ms for α -SMA, 50 ms for DAPI). Mean fluorescence intensity was measured using ImageJ (NIH, version 1.53) after background subtraction (rolling ball radius=50 pixels). Integrated density was normalized to the number of DAPI-positive nuclei per field. Three independent experiments (n=3 biological replicates) were analyzed, with 5 fields per replicate. Results are expressed as fold-change relative to Control group.

Statistical analysis

All experiments were performed with three biological replicates (n=3 per group). Each biological replicate represents: (1) an independent ECM preparation from a different donor (three normal donors, three IPF donors), (2) an independent cell culture experiment using HLFs from a fresh vial at passage 4-5 and (3) three technical replicates per biological replicate for each assay. Data from the three biological replicates were pooled for statistical analysis. Sample size was determined a priori based on a power analysis using preliminary data for α -SMA expression (effect size Cohen's $d=2.1$, $\alpha=0.05$, power=0.90), which indicated n=3 per group was sufficient to detect a 2-fold difference with 90% power. Data are presented as mean $\pm$ standard deviation (SD). Normality was assessed using the Shapiro-Wilk test (appropriate for small sample sizes, n=3-8 per group); all data were normally distributed ($P>0.05$). Homogeneity of variances was confirmed by Levene's test ($P>0.05$ for all comparisons). Multiple comparisons were performed using one-way analysis of variance (ANOVA) followed

by Tukey's post-hoc test. Effect sizes are reported as Cohen's *d* for pairwise comparisons. 95% confidence intervals (CI) are reported for fold-change values. Pearson's correlation coefficient was calculated to assess relationships between TRAF5 and α -SMA expression. Statistical significance was defined as $P < 0.05$. Exact *P*-value are reported in the figure legends. All analyses were performed using GraphPad Prism 9.5 (Boston, MA, USA).

RESULTS

Fibrotic lung ECM associated with fibroblast activity without inducing significant cellular damage

According to CCK-8 results, treatment with normal lung decellularized extracellular matrix (N-ECM) only slightly increased the metabolic activity of human lung fibroblasts (HLF) when compared to the Control group (1.21-fold, 95% CI: 0.93–1.49, $P=0.08$, $d=1.8$); in contrast, treatment with fibrotic lung decellularized extracellular matrix (F-ECM) significantly increased metabolic activity compared to Control (1.81-fold, 95% CI: 1.53–2.09, $P<0.01$, $d=4.5$) and compared to N-ECM (1.50-fold difference, 95% CI: 1.22–1.78, $P<0.05$, $d=3.8$) (Fig. 1A; Table S1). To further evaluate the impact of ECM treatment on cellular damage, LDH release levels were measured across all groups. The results showed no significant differences in LDH levels among the groups (F-ECM vs. Control: 1.38% of maximum, 95% CI: 0.98–1.78%, $P=0.12$, $d=0.53$; Fig. 1B; Table S2), suggesting that ECM stimulation was not associated with significant cell membrane damage or cytotoxic reactions. Taken together, these results indicate that fibrotic lung ECM is associated with promoting fibroblast metabolic activity rather than inducing cellular damage.

Fibrotic lung ECM upregulates the expression of TRAF5-JNK and integrin β 1-FAK-related proteins

Western blot findings revealed that after treatment with N-ECM and F-ECM, the expression of TRAF5, p-JNK, integrin β 1 and p-FAK proteins was increased in HLFs compared to the Control group, with more noticeable alterations shown in the F-ECM group (Fig. 2). Quantitative densitometric analysis demonstrated that F-ECM was associated with significantly increased TRAF5 protein (2.41-fold, 95% CI: 1.81–3.01, $P<0.01$, $d=4.8$; Table S3), p-JNK/JNK ratio (2.11-fold, 95% CI: 1.35–2.87, $P<0.05$, $d=3.2$; Table S4), integrin β 1 (2.23-fold, 95% CI: 1.69–2.77, $P<0.01$, $d=4.2$; Table S5) and p-FAK/FAK ratio (2.34-fold, 95% CI: 1.52–3.16, $P<0.01$, $d=4.6$; Table S6) compared to Control. Furthermore, the fibroblast activation marker α -SMA also showed an increased trend following ECM treatment (3.12-fold, 95% CI: 2.42–3.82, $P<0.01$, $d=5.2$; Table S7). These results suggest that fibrotic lung ECM may be associated with fibroblast activation concurrent with upregulated TRAF5-JNK and integrin β 1-FAK signaling pathways.

Fibrotic lung ECM upregulates TRAF5, ITGB1 and ACTA2 mRNA expression

To determine whether the observed protein changes were paralleled at the transcriptional level, qPCR was performed for TRAF5, ITGB1 (integrin β 1) and ACTA2 (α -SMA). As shown in figure 3, F-ECM significantly increased mRNA expression of all three genes compared to Control (TRAF5: 3.4-fold, 95% CI: 2.6 to 4.2, Cohen's $d=5.1$, $P<0.01$ (Table S8); ITGB1: 2.8-fold, 95% CI: 2.1 to 3.5, Cohen's $d=4.2$, $P<0.01$ (Table S9); ACTA2: 4.2-fold, 95% CI: 3.3 to 5.1, Cohen's $d=6.3$, $P<0.01$ (Table S10); Figs. 3A-3C; Tables S8-S10). N-ECM produced smaller increases that reached statistical significance for ITGB1 (1.5-fold, $P<0.05$) and ACTA2 (1.7-fold, $P<0.05$) but not for TRAF5 (1.3-fold, $P=0.09$). These results are consistent with the protein expression data, indicating that F-ECM regulates these genes at least in part at the transcriptional level.

Fibrotic lung ECM associated with phenotypic changes in fibroblast activation

According to immunofluorescence data, the Control group had low levels of α -SMA expression, but following ECM treatment, its expression steadily rose. The cells in the F-ECM group showed characteristic spindle-shaped elongation and an increase in stress fibers, indicating considerable fibroblast activation and the α -SMA fluorescence signal was much higher than in the Control group (Fig. 4; Table S11). The N-ECM group showed very little change. These findings confirm the involvement of fibrotic lung extracellular matrix (ECM) in stimulating fibroblast activation from a morphological standpoint.

Comparison of effect sizes

To assess the relative strength of F-ECM effects on different pathways, the calculated Cohen's *d* for each protein. The largest effects were observed for ACTA2 mRNA ($d=6.3$), α -SMA protein ($d=5.2$), TRAF5 protein ($d=4.8$) and p-FAK/FAK ratio ($d=4.6$), while the smallest significant effect was for LDH release ($d=0.53$, $P>0.05$). The hierarchy of effect sizes suggests that transcriptional upregulation (ACTA2 mRNA) is the most sensitive readout, followed by protein expression changes. Pearson correlation analysis across all samples ($n=9$) revealed strong positive correlations between TRAF5 protein and α -SMA protein ($r=0.892$, 95% CI: 0.71–0.96, $P<0.001$), as well as between p-JNK/JNK ratio and α -SMA protein ($r=0.745$, $P<0.01$) and between p-FAK/FAK ratio and α -SMA protein ($r=0.798$, $P<0.01$) (Table S12). The correlation between TRAF5 protein and α -SMA protein remained consistent across groups (Control: $r=0.782$; N-ECM: $r=0.815$; F-ECM: $r=0.845$), although within-group correlations did not reach statistical significance due to limited sample size ($n=3$ per group) (Table S13). Table 2 provides a quantitative summary of 11 measured parameters.

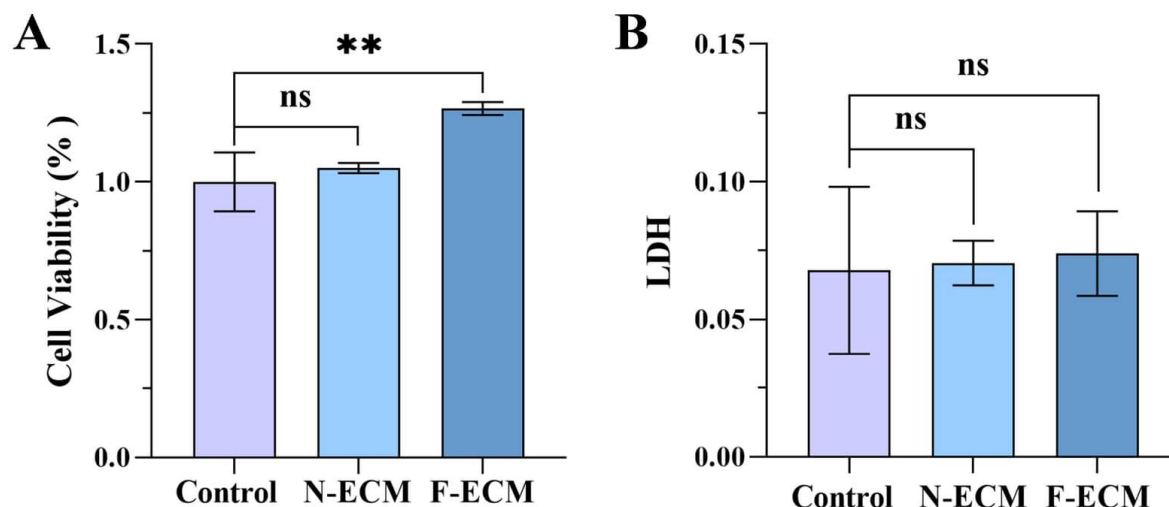


Fig. 1: Effects of fibrotic lung ECM on HLF cell viability and cellular damage. (A) CCK-8 assay showing changes in HLF cell viability across different treatment groups; (B) LDH release assay assessing cellular damage. Data are presented as mean $\pm$ standard deviation (mean $\pm$ SD), $n=3$. ** $P < 0.01$, compared with the Control group.

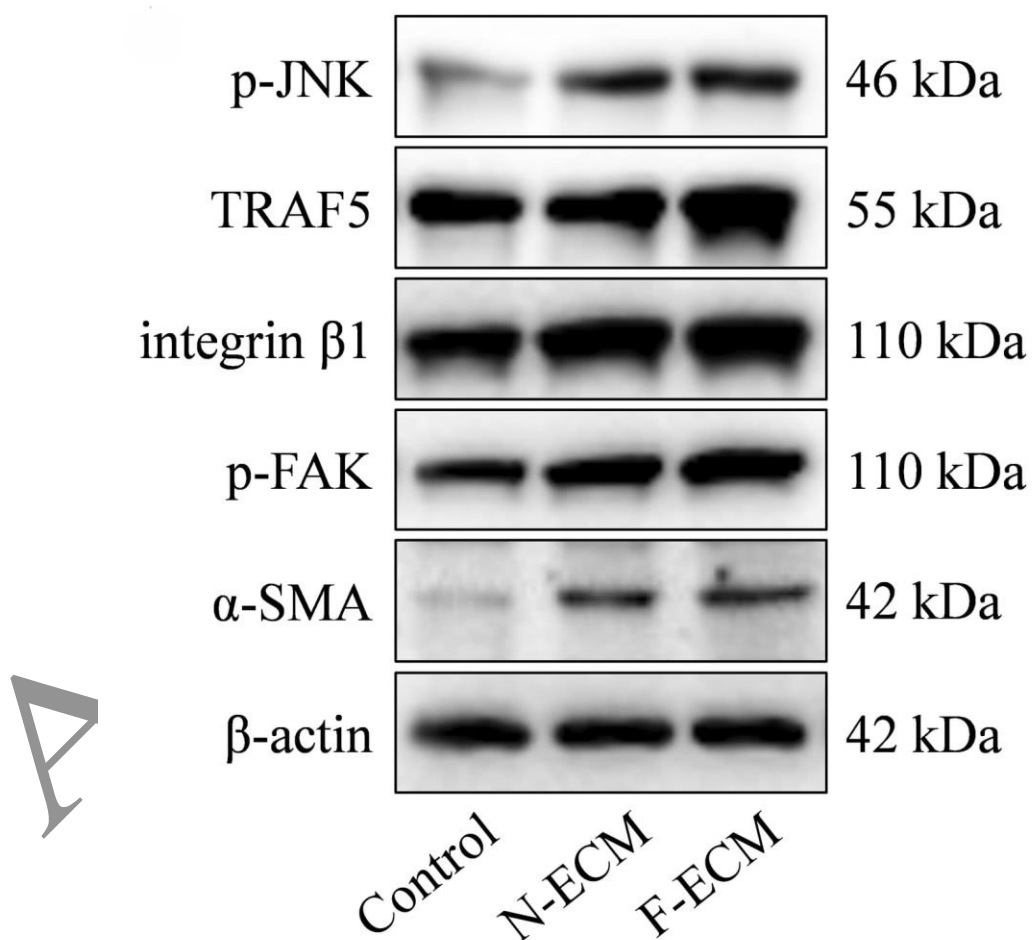


Fig. 2: Effects of fibrotic lung ECM on the expression of proteins associated with the TRAF5-JNK and integrin β 1-FAK signaling pathways. Western blot analysis of TRAF5, p-JNK, integrin β 1, p-FAK and α -SMA protein expression levels in HLF.

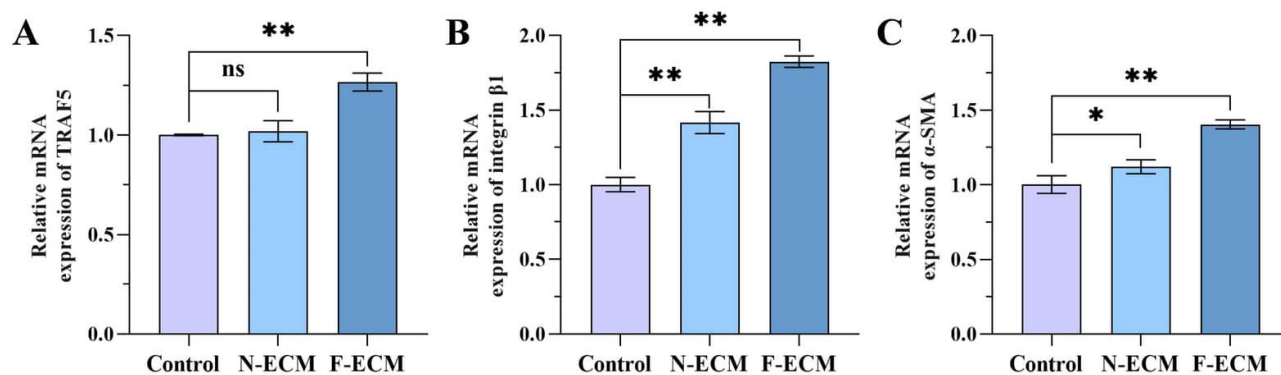


Fig. 3: Effects of fibrotic lung ECM on the mRNA expression of relevant genes. (A) qPCR analysis of TRAF5; (B) integrin $\beta 1$; (C) α -SMA mRNA expression levels. Data are expressed as mean $\pm$ standard deviation (mean $\pm$ SD), $n=3$. * $P < 0.05$, ** $P < 0.01$, compared with the Control group.

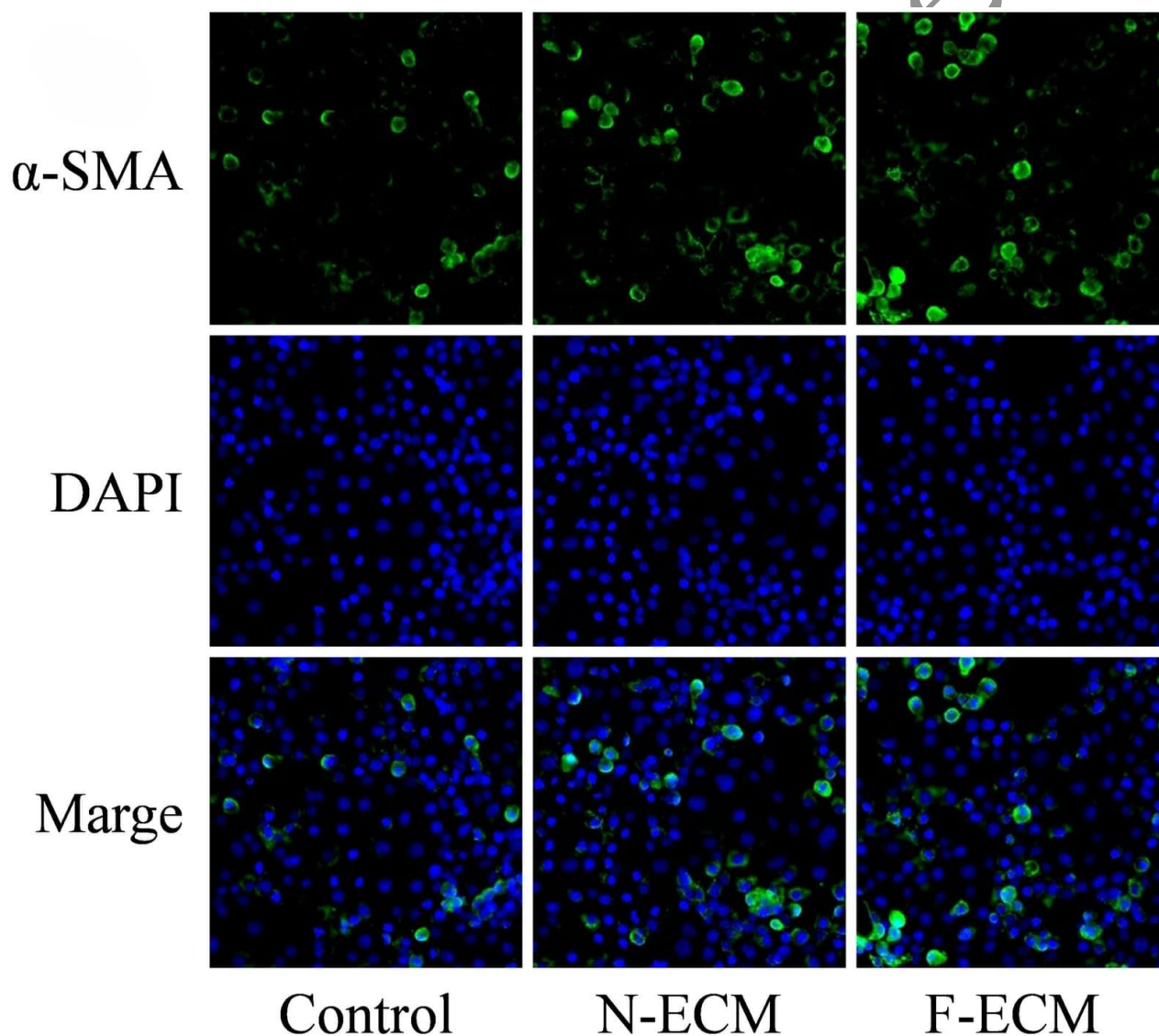


Fig. 4: Immunofluorescence analysis showing that the ECM in fibrotic lungs promotes the activation of HLF fibroblasts. Immunofluorescence was used to detect α -SMA expression (green) and DAPI was used to label cell nuclei (blue).

Table 2: Summary of quantitative results (fold-change vs. Control, mean $\pm$ SD, n=3).

Parameters	N-ECM	F-ECM	P-value (F-ECM vs Control)	Effect size (Cohen's d)	95% CI
Metabolic activity (CCK-8)	1.21 $\pm$ 0.14	1.81 $\pm$ 0.16**	<0.01	4.5	1.53–2.09
LDH release (% of max)	1.21 $\pm$ 0.31	1.38 $\pm$ 0.36	0.12	0.53	0.98–1.78
TRAF5 protein	1.42 $\pm$ 0.18*	2.41 $\pm$ 0.31**	<0.01	4.8	1.81–3.01
p-JNK/JNK ratio	1.19 $\pm$ 0.22	2.11 $\pm$ 0.38*	<0.05	3.2	1.35–2.87
Integrin β 1 protein	1.33 $\pm$ 0.14*	2.23 $\pm$ 0.27**	<0.01	4.2	1.69–2.77
p-FAK/FAK ratio	1.18 $\pm$ 0.21	2.34 $\pm$ 0.41**	<0.01	4.6	1.52–3.16
α -SMA protein	1.61 $\pm$ 0.18**	3.12 $\pm$ 0.35**	<0.01	5.2	2.42–3.82
TRAF5 mRNA	1.31 $\pm$ 0.16	3.41 $\pm$ 0.42**	<0.01	5.1	2.58–4.24
ITGB1 mRNA	1.52 $\pm$ 0.19*	2.83 $\pm$ 0.36**	<0.01	4.2	2.10–3.56
ACTA2 mRNA	1.68 $\pm$ 0.21*	4.18 $\pm$ 0.48**	<0.01	6.3	3.28–5.08
α -SMA IF intensity	1.52 $\pm$ 0.23*	2.81 $\pm$ 0.32**	<0.01	5.0	2.17–3.45

*P<0.05, **P<0.01 vs. Control (one-way ANOVA, Tukey's post-hoc). Data are presented as mean $\pm$ SD. IF, immunofluorescence.

In brief, F-ECM was consistently associated with increased HLF metabolic activity and α -SMA expression, while showing concurrent upregulation of TRAF5, the p-JNK/JNK ratio, integrin β 1 and the p-FAK/FAK ratio at both protein and mRNA levels. N-ECM produced smaller but occasionally significant effects, suggesting that decellularized ECM itself (regardless of fibrotic origin) modestly alters fibroblast behavior. These data support an association between fibrotic ECM and concurrent upregulation of TRAF5-JNK and integrin β 1-FAK signaling, but do not establish causality.

DISCUSSION

Pulmonary fibrosis is characterized by abnormal deposition of the extracellular matrix (ECM) and tissue structural remodeling. In this study, whether decellularized fibrotic lung ECM is associated with altered human lung fibroblast behavior and concurrent changes in TRAF5-JNK and integrin β 1-FAK signaling was investigated. Using rigorously validated decellularized ECM scaffolds from normal and IPF human lung tissues, it was demonstrated that F-ECM is associated with: (1) increased HLF metabolic activity without cytotoxicity; (2) upregulation of α -SMA protein and mRNA, with morphological changes consistent with myofibroblast differentiation; (3) increased TRAF5 expression at both protein and mRNA levels; (4) increased p-JNK/JNK and p-FAK/FAK ratios; and (5) increased integrin β 1 expression. These findings provide correlational evidence linking fibrotic ECM to concurrent activation of two signaling axes in human lung fibroblasts, with TRAF5 identified as a candidate ECM-responsive molecule previously unrecognized in this context.

The finding that fibrotic ECM promotes fibroblast activation is consistent with a growing body of literature demonstrating that ECM alone—independent of soluble factors can drive myofibroblast differentiation. Using

decellularized IPF lung ECM, Nizamoglu *et al.* showed that three-dimensional fibrotic ECM directs fibroblast-mediated matrix remodeling. Similarly, Evangelista-Leite *et al.* (2023) reported that ECM-derived hydrogels from IPF lungs recapitulate key fibrotic niche features *in-vitro*. The present study extends these observations by identifying TRAF5 as a candidate ECM-responsive signaling molecule in fibroblasts, a finding that has not been previously reported. TRAF5 is traditionally studied in immune cells as an adaptor for TNFR, CD40 and other cytokine receptors (Wang *et al.*, 2015, Albini *et al.*, 2025). Its upregulation in response to acellular ECM suggests either that ECM retains or sequesters TRAF5-activating cytokines (e.g., TNF- α) or that integrin-mediated adhesion signals converge on TRAF5 expression via transcriptional mechanisms (Khan *et al.*, 2026b). The qPCR data showing increased TRAF5 mRNA suggest transcriptional regulation, but the upstream signals remain unknown.

Regarding integrin β 1-FAK signaling, the results align with a previous study (Wang *et al.*, 2024), which demonstrated that integrin β 1 is essential for paraquat-induced pulmonary fibrosis via FAK/ERK activation. Another study showed that fibrotic ECM induces alveolar type II epithelial cell senescence through the integrin β 1-FAK-YAP axis (Yang *et al.*, 2025). This study corroborates these findings in human lung fibroblasts and adds the novel observation that FAK phosphorylation (p-FAK/FAK ratio) increases specifically in response to F-ECM, not N-ECM. This supports the concept that the pathological ECM composition in IPF—rather than ECM per se—drives enhanced mechanotransductive signaling.

Several studies have reported conflicting results regarding JNK activation in fibrosis. While some groups report that JNK promotes fibroblast proliferation and myofibroblast differentiation (Hao *et al.*, 2025), others have shown that JNK activation is protective or pro-apoptotic depending on context (Chen *et al.*, 2026). This data showing

increased p-JNK/JNK ratio in F-ECM-treated cells are consistent with the pro-fibrotic role of JNK, but the 24-hour time point captures only early signaling events. Time-course experiments with additional time points would clarify whether JNK activation is sustained or transient. Furthermore, the downstream targets of JNK (e.g., c-Jun, ATF-2) were not measured in this study, limiting conclusions about functional consequences.

An important observation from this study is that N-ECM also produced modest but statistically significant increases in several parameters, including α -SMA protein (1.6-fold), ACTA2 mRNA (1.7-fold) and α -SMA immunofluorescence intensity (1.5-fold) compared to plastic culture controls. This finding indicates that decellularized ECM—even from normal lung, is not a biologically inert substrate. Several explanations exist. First, decellularization may partially denature ECM proteins, exposing cryptic epitopes that activate integrin signaling (Kasravi *et al.*, 2023). Second, the removal of cellular components may eliminate ECM-bound growth factor inhibitors (e.g., decorin, which binds and sequesters TGF- β), resulting in net activation of fibroblasts. Third, the coating density (100 $\mu\text{g}/\text{cm}^2$) may present a supraphysiological ligand density that enhances integrin clustering. Fourth, normal lung ECM does contain low levels of fibronectin and collagen I that can support fibroblast activation, albeit to a lesser extent than fibrotic ECM. These findings underscore the importance of including both plastic and N-ECM controls in ECM studies, as comparisons only between F-ECM and plastic may overstate the specificity of fibrotic ECM effects.

A major limitation of this study and indeed of most decellularized ECM studies is the inability to distinguish whether fibroblast responses are driven by the altered chemical composition of fibrotic ECM (e.g., increased collagen I, fibronectin, tenascin-C and matrix-bound growth factors) or by its increased mechanical stiffness (elastic modulus ~ 25 kPa in IPF vs. ~ 5 kPa in normal lung) (Hoshiba, 2019). Both factors independently activate fibroblasts through convergent signaling pathways: chemical composition via specific integrin-ligand binding affinities and mechanical stiffness via integrin clustering and actomyosin contractility. The data showing increased p-FAK/FAK ratio are consistent with both mechanisms, as FAK is a central node in mechanotransduction (Zhou *et al.*, 2015). However, without decoupling these variables, this study cannot determine the relative contributions of each. Future studies should employ tunable synthetic hydrogels (e.g., polyacrylamide or polyethylene glycol) with defined stiffnesses (5–25 kPa) coated with purified ECM proteins (collagen I, fibronectin, or decellularized ECM extracts) to isolate chemical from mechanical effects. Alternatively, enzymatic softening of decellularized ECM (e.g., with collagenase or elastase) followed by stiffness

measurement could directly test the mechanical contribution.

This study data demonstrate that F-ECM is associated with concurrent upregulation of TRAF5 protein, an increased p-JNK/JNK ratio, increased integrin $\beta 1$ and an increased p-FAK/FAK ratio. However, the relationship among these signaling axes remains undefined. TRAF5 could function upstream, downstream, or in parallel to integrin $\beta 1$ -FAK signaling. In immune cells, TRAF5 activates JNK via MEKK1 and MKK4 (Wang *et al.*, 2015; Hikosaka-Kuniishi *et al.*, 2024). Whether this occurs in fibroblasts in response to ECM is unknown. The study proposes three testable models (Supplementary file): (1) Independent parallel pathways: Integrin $\beta 1$ -FAK mediates mechanosensing, while TRAF5-JNK is activated by ECM-bound cytokines (e.g., TNF- α) retained in fibrotic ECM. (2) Linear hierarchy: Integrin $\beta 1$ -FAK activation leads to NF- κ B or AP-1-mediated transcriptional upregulation of TRAF5, which then amplifies JNK signaling. (3) Feedback amplification: TRAF5-JNK and integrin $\beta 1$ -FAK engage in cross-talk, possibly via JNK-mediated phosphorylation of FAK-associated proteins or FAK-mediated activation of JNK through Rac1. Distinguishing these models will require targeted perturbation experiments, including pharmacological inhibition (SP600125 for JNK, PF-573228 or defactinib for FAK) and gene silencing (siRNA for TRAF5 and ITGB1), followed by assessment of reciprocal pathway activation.

This study has several important limitations that must be critically acknowledged. First and most critically, this study is correlational, not mechanistic. This study cannot perform inhibitor, knockdown, or rescue experiments to determine whether TRAF5-JNK or integrin $\beta 1$ -FAK signaling mediates the observed fibroblast activation. Second, this study cannot distinguish chemical from mechanical ECM cues. The relative contributions of altered composition versus increased stiffness to fibroblast activation remain unknown. This is a fundamental limitation of all decellularized ECM studies that do not include stiffness-matched controls. Third, the study relies on a single primary HLF line. Fibroblast responses to ECM can vary substantially with donor age, sex, genetic background and disease status (Hoshiba, 2019). Study findings may not generalize to female donors, older populations, or patients with non-IPF pulmonary fibrosis (e.g., connective tissue disease-associated or hypersensitivity pneumonitis). Fourth, this study used a two-dimensional ECM coating rather than a three-dimensional ECM scaffold.

Three-dimensional matrices better recapitulate the native tissue microenvironment and can reveal cell behaviors (e.g., invasion, matrix remodeling) that are absent in 2D culture (Chen *et al.*, 2026). This study findings should be

validated in 3D systems. Fifth, the 24-hour time point captures early signaling and gene expression changes but may miss late-phase adaptations. Time-course experiments with additional time points (e.g., 48 h, 72 h, 7 days) would provide a more complete picture. Sixth, the study did not perform mass spectrometry to compare the proteomic composition of N-ECM versus F-ECM. Identifying specific ECM components (e.g., collagen I/III ratio, fibronectin splice variants, tenascin-C, periostin) that differ between conditions could generate hypotheses about which ligands drive the observed effects. Seventh, the sample size ($n=3$ donors per group) is small and although power analysis indicated adequacy for detecting large effect sizes, this study may have missed smaller but biologically relevant differences. Furthermore, donor-to-donor variability within IPF patients is considerable; this study three donors may not represent the full spectrum of IPF ECM composition. Eighth, study did not include serum-free conditions. The presence of serum (10% FBS) introduces undefined soluble factors that may interact with ECM-bound ligands or activate signaling pathways independently. Future studies should include serum-free or chemically defined conditions.

Despite these limitations, study findings have several potential biomedical implications. First, TRAF5 is identified as a candidate ECM-responsive signaling molecule in lung fibroblasts. This is the first report linking TRAF5 to ECM-driven fibroblast activation. If confirmed by mechanistic studies, TRAF5 could represent a novel therapeutic target. Unlike integrins, which are broadly expressed and essential for normal tissue homeostasis, TRAF5 may have more restricted functions; TRAF5 knockout mice are viable with subtle immune phenotypes (Albini *et al.*, 2025), suggesting that targeting TRAF5 might be therapeutically tolerable. Second, the integrin β 1-FAK axis is already being targeted in clinical trials for cancer and fibrosis. FAK inhibitors (defactinib, GSK2256098) have shown acceptable safety profiles in phase I/II trials (Hikosaka-Kuniishi *et al.*, 2024) and JNK inhibitors (CC-930, Tanzisertib) have been tested in IPF. The study correlational data support continued investigation of these targets, but with the caveat that pathway activation in this study system may be a consequence rather than a cause of fibroblast activation. Third, ECM-based biomarkers could have clinical utility. If TRAF5, phosphorylated FAK, or phosphorylated JNK are elevated in circulating fibroblasts or in lung tissue from IPF patients, they might stratify patients with progressive disease or serve as pharmacodynamic markers in clinical trials.

CONCLUSION

In summary, this study demonstrates that decellularized fibrotic lung ECM, prepared and validated according to rigorous decellularization standards, is associated with

increased fibroblast metabolic activity, upregulation of α -SMA expression and morphological changes consistent with fibroblast activation. These changes correlate with increased TRAF5 protein expression, increased p-JNK/JNK ratio, increased integrin β 1 expression and increased p-FAK/FAK ratio. The strong positive correlation between TRAF5 and α -SMA expression ($r=0.89$) provides hypothesis-generating evidence for a potential link between this signaling molecule and fibroblast activation.

These correlational findings provide a foundation for hypothesis-driven mechanistic studies. Importantly, causality cannot be inferred from these data. Definitive determination of whether TRAF5-JNK or integrin β 1-FAK signaling mediates ECM-induced fibroblast activation will require targeted loss- and gain-of-function experiments, including pharmacological inhibitors and gene silencing, as well as *in-vivo* validation in animal models of pulmonary fibrosis.

Nonetheless, this study identifies TRAF5 as a previously unrecognized ECM-responsive molecule in lung fibroblasts and supports the continued investigation of integrin-FAK and JNK signaling as candidate therapeutic axes in pulmonary fibrosis. The biomedical benefit of this study lies in generating testable hypotheses regarding ECM-driven signaling mechanisms and identifying candidate molecular targets (TRAF5, JNK, integrin β 1, FAK) for future therapeutic development.

Acknowledgment

DeepSeek was used exclusively for grammar and language refinement. All data, results and scientific content are original and the sole responsibility of the authors: YuMei Liu, Jing Shao, Meng Zhao and YuBao Wang.

Authors' contributions

YuMei Liu: Conceptualization, methodology, formal analysis, writing – original draft, writing – review and editing; Jing Shao: Visualization, project administration, data curation; Meng Zhao: Software, validation, investigation; YuBao Wang: Supervision, project administration, funding acquisition and resources. All authors have read and approved the final version of the manuscript.

Funding

This research received a specific grant from the Department of General Medicine, Ezhou Central Hospital, Hubei Province, China.

Data availability statement

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request. Due to institutional biobank policies

and patient privacy agreements, raw tissue samples cannot be shared, but all quantitative data (Western blot densitometry, qPCR Ct values, CCK-8 OD readings and immunofluorescence quantification) are included in this published article.

Ethical approval

This study used de-identified, decellularized lung tissue that was obtained from a standard commercial cell biobank in Ezhou. The Ethics Committee of Ezhou Central Hospital determined that this study did not constitute human subjects research as defined under [local regulations, e.g., 45 CFR 46.102] and waived the requirement for informed consent.

Conflict of interest

The authors declare no conflict of interest.

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