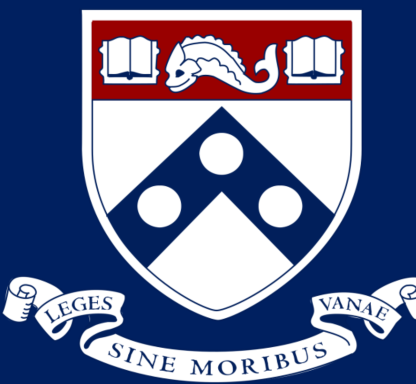
Impact of Targeted Inhibition of TGFβ Receptor on TGFβ-induced Fibroblast Differentiation and Matrix Remodeling



Rhea Bakshi, Leslie A Hopper, Zebin Xiao, Pranidhi Baddam, Li Huang, Ellen Puré*

Department of Biomedical Sciences, University of Pennsylvania, Philadelphia, Pennsylvania, 19104, US

Introduction

The tumor microenvironment is comprised of a variety of components, most notably the extracellular matrix (ECM), stromal cells such as cancer-associated fibroblasts (CAFs), and immune cells[1]. Communication between cells in the tumor microenvironment and the ECM leads to the release of soluble factors, such as TGFβ, that trigger stromal cells that contribute to ECM remodeling and are characterized by their immunosuppressive qualities[2].

Myofibroblasts are a sub-population of cancer-associated fibroblasts (CAFs) in the tumor stroma that specialize in ECM secretion and contraction[3]. Specifically, myofibroblasts are α-smooth muscle actin-positive cells that form stress-fiber bundles and play a key role in driving fibrosis. Myofibroblast activation and fibrosis is associated with the increased production of multiple components of the ECM – including type I fibrillar collagen and fibronectin[4]. Hence, there is a noteworthy relationship between ECM structure and the development/function of myofibroblasts that might serve as an attractive target for therapeutic treatments in some contexts.

Fibroblast activation protein (FAP) is a type II transmembrane serine protease that is expressed in a subset of CAFs in the tumor stroma[5]. FAP has been found to participate in the progression of malignant tumors and hence, is being investigated as a therapeutic target in cancer treatment[6]. Transforming Growth Factor β (TGFβ) is a pleiotropic factor that drives inflammation and cancer in part by targeting stromal cells. TGFβ receptors are classified into 2 subtypes – type I and type II. The activation of type II and subsequent phosphorylation of type I receptor kinase initiates a cascade of downstream substrate and regulatory protein activation[7]. The TGFβ signaling pathways in cancer cells are often mutated, causing an increase in TGFβ production and activity. This upregulation in TGFβ results in TGFβ acting upon stromal cells to suppress immune function and increase the proliferation of fibroblasts[8]. FAP and TGFβ have both been discovered to be upregulated in solid tumors. Recent evidence has suggested that TGFβ regulates FAP expression in the tumor stroma[6].

In this work, I investigated the effects of TGFβ on the phenotypic differentiation of fibroblasts during production of fibroblast-derived matrices (FDMs) using a primary human lung fibroblast cell line, CCD19 Lu. In order to downregulate the functionality of TGFβ, I used a bispecific antibody that has one arm specific for TGFβRII and the other arm specific for FAP. We targeted TGFβRII, not TGFβ directly, due to its significant downstream effects on other essential biological processes. This bispecific antibody preferentially bound to the TGFβRII on FAP+ cells, which allowed me to investigate its effects on a very specific subset of fibroblasts.

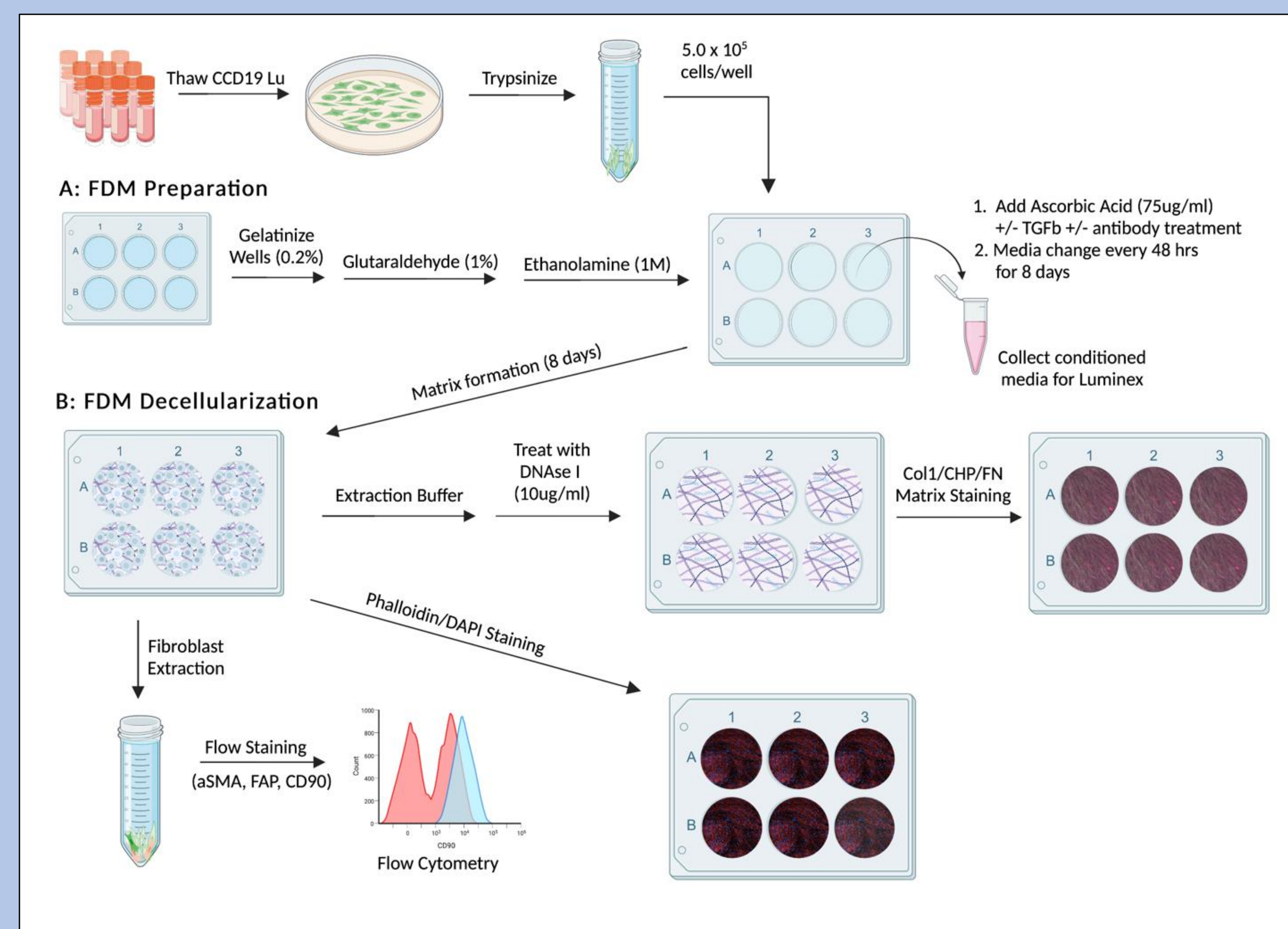


Figure 1. General Experimental Layout (created with BioRender.com)

References

[1]Khalaf K, Hana D, Chou JT, Singh C, Mackiewicz A, Kaczmarek M. Aspects of the Tumor Microenvironment Involved in Immune Resistance and Drug Resistance. Front Immunol. 2021 May 27;12:656364. doi: 10.3389/fimmu.2021.656364. PMID: 34122412; PMCID: PMC8190405.

[2]Kano, A. Tumor cell secretion of soluble factor(s) for specific immunosuppression. Sci Rep 5, 8913 (2015). https://doi.org/10.1038/srep08913

[3]Tai Y, Woods EL, Dally J, Kong D, Steadman R, Moseley R, Midgley AC. Myofibroblasts: Function, Formation, and Scope of Molecular Therapies for Skin Fibrosis. Biomolecules. 2021 Jul 23;11(8):1095. doi: 10.3390/biom11081095. PMID: 34439762; PMCID: PMC8391320.

[4]Klingberg F, Hinz B, White ES. The myofibroblast matrix: implications for tissue repair and fibrosis. J Pathol. 2013 Jan;229(2):298-309. doi: 10.1002/path.4104. PMID: 22996908; PMCID: PMC4005341.

[5]Xin L, Gao J, Zheng Z, Chen Y, Lv S, Zhao Z, Yu C, Yang X and Zhang R (2021) Fibroblast Activation Protein- α as a Target in the Bench-to-Bedside Diagnosis and Treatment of Tumors: A Narrative Review. Front. Oncol. 11:648187. doi: 10.3389/fonc.2021.648187

[6]Krepela E, Vanickova Z, Hrabal P, Zubal M, Chmielova B, Balaziova E, Vymola P, Matrasova I, Busek P, Sedo A. Regulation of Fibroblast Activation Protein by Transforming Growth Factor Beta-1 in Glioblastoma Microenvironment. Int J Mol Sci. 2021 Jan 21;22(3):1046. doi: 10.3390/ijms22031046. PMID: 33494271; PMCID: PMC7864518.

[7]Massagué J. TGFβ signaling in context. Nat Rev Mol Cell Biol. 2012 Oct;13(10):616-30. doi: 10.1038/nrm3434. Epub 2012 Sep 20. PMID: 22992590; PMCID: PMC4027049.

[8]Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. N Engl J Med. 2000 May 4;342(18):1350-8. doi: 10.1056/NEJM200005043421807. PMID: 10793168.

Results

1. TGFβ drives FAP+αSMAhi fibrogenic transitional myofibroblast phenotype in FDM-generating conditions.

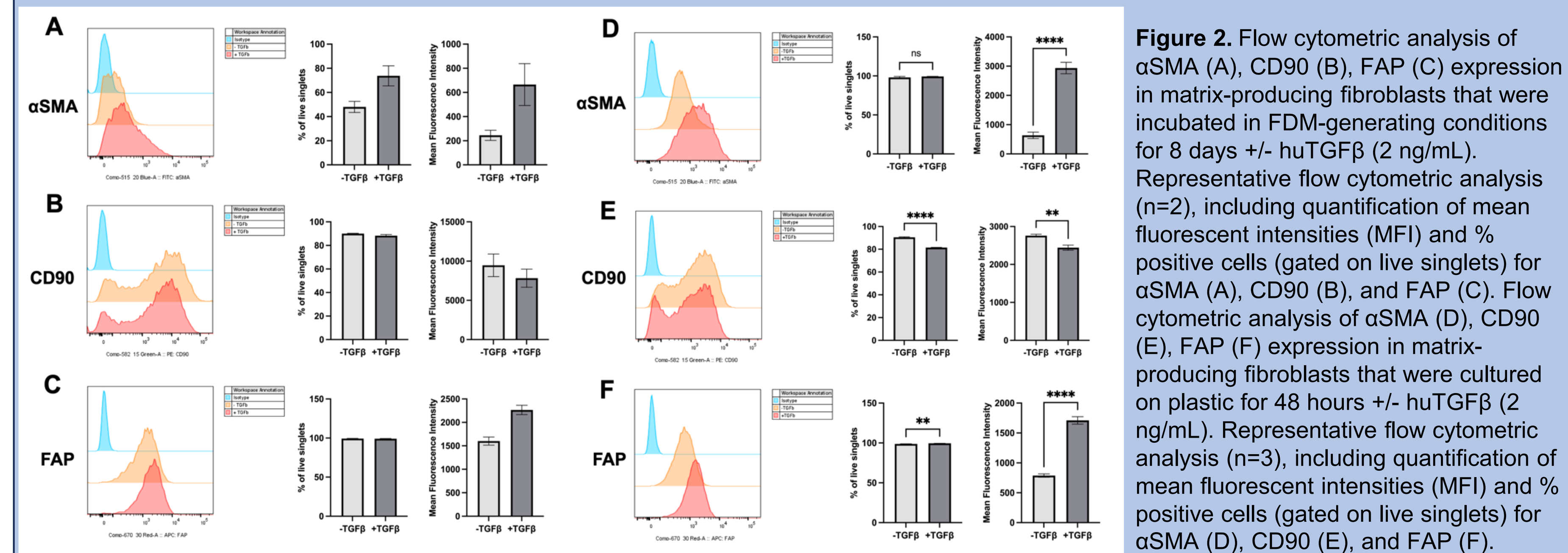


Figure 2. Flow cytometric analysis of αSMA (A), CD90 (B), FAP (C) expression in matrix-producing fibroblasts that were incubated in FDM-generating conditions for 8 days +/- huTGFβ (2 ng/mL). Representative flow cytometric analysis (n=2), including quantification of mean fluorescent intensities (MFI) and % positive cells (gated on live singlets) for αSMA (A), CD90 (B), and FAP (C). Flow cytometric analysis of αSMA (D), CD90 (E), FAP (F) expression in matrix-producing fibroblasts that were cultured on plastic for 48 hours +/- huTGFβ (2 ng/mL). Representative flow cytometric analysis (n=3), including quantification of mean fluorescent intensities (MFI) and % positive cells (gated on live singlets) for αSMA (D), CD90 (E), and FAP (F).

2. TGFβ induces increased cytoskeletal stress fiber formation and production of a more fibrotic-like matrix.

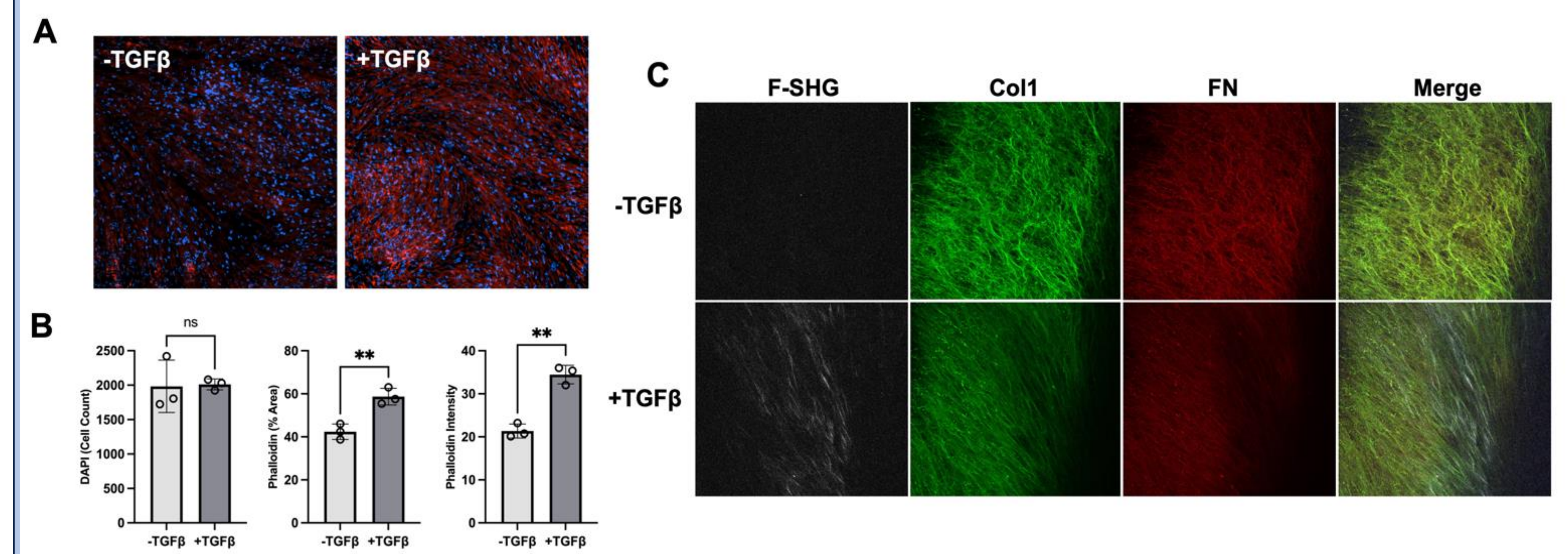


Figure 3. (A) Representative phalloidin staining of the actin cytoskeleton in matrix-producing fibroblasts that were incubated in FDM-generating conditions for 8 days +/- huTGFβ (2 ng/mL). (B) Quantification of cell nuclei (DAPI), actin filaments (% area phalloidin), and phalloidin staining, obtained using *ImageJ* software. (F) Representative IF staining of FN, Col1, CHP, and two-photon second harmonic generation imaging of fibrillar collagen in decellularized FDMs generated by CCD19 human lung fibroblasts.

3. The fibrogenic response driven by TGFβ can be partially inhibited by the addition of bispecific antibodies targeting FAP and TGFβRII.

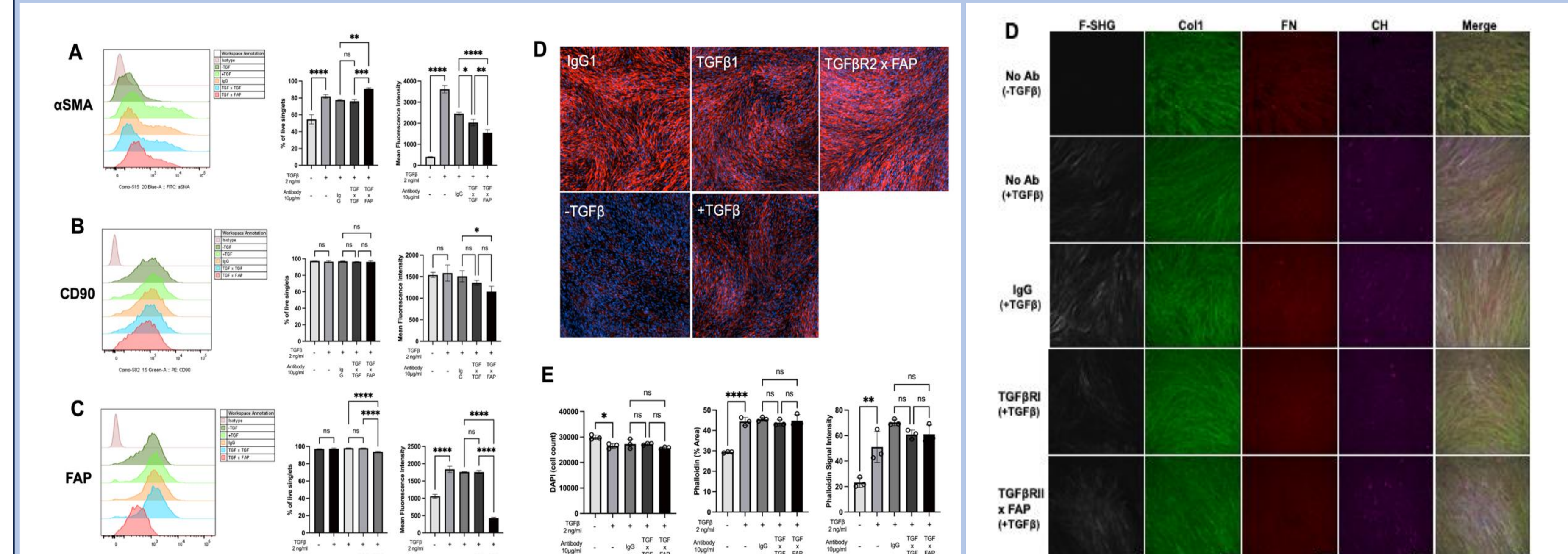


Figure 4. Flow cytometric analysis of αSMA (A), CD90 (B), FAP (C) expression in matrix-producing fibroblasts that were incubated in FDM-generating conditions for 8 days +/- huTGFβ (2 ng/mL) and bispecific antibody treatment (10 ug/mL). Representative flow cytometric analysis, including quantification of mean fluorescent intensities (MFI) and % positive cells (gated on live singlets) for αSMA (A), CD90 (B), and FAP (C) . (D) Representative phalloidin staining of the actin cytoskeleton in matrix-producing fibroblasts following treatment with bispecific antibody. (E) Quantification of cell nuclei, actin filaments (% area phalloidin), and phalloidin staining intensity present in each condition, obtained using *ImageJ* software. (F) Representative 10x IF staining of FN, Col1, CHP, and two-photon second harmonic generation imaging of fibrillar collagen in decellularized FDMs generated by CCD19 human lung fibroblasts (scale bar = 100 uM)

Materials & Methods

Cell Culture

The primary human lung fibroblast cell line, CCD19-Lu, used in these experiments was purchased from ATCC. The CCD19 lung fibroblasts were cultured in 10% FCS EMEM (supplemented with non-essential amino acids, sodium pyruvate, penicillin-streptomycin, and L-glutamine) and grown on tissue culture-treated plastic.

Fibroblast-Derived Matrix Production

The production of fibroblast-derived matrices was conducted using the methods outlined by Dr. Edna Cukierman. Fibroblast-derived matrices were generated in both 6-well plates and 35-mm dishes. Plates and dishes were coated gelatin and incubated at 37°C for 1 hr. Dishes were rinsed with PBS, then incubated with 1% glutaraldehyde in PBS for 30 min at RT to cross-link gelatin. Dishes were washed with PBS prior to addition of 1M ethanolamine in PBS or 30 min at RT to block any remaining glutaraldehyde. Ethanolamine was aspirated and dishes were rinsed to ensure the removal of any trace amounts of ethanolamine. P4 CCD19-Lu fibroblasts were trypsinized, counted and resuspended in 10% FCS EMEM. The resulting cell suspension was added to the gelatin-coated dishes/plates at a density of $5x10^5$ cells per well. Upon confirmation of confluency, media was collected and replaced with 10% FCS EMEM containing freshly prepared 75 µg/mL L-ascorbic acid, and +/- 2 ng/mL TGFβ (based on experimental condition). For samples that received pre-treatment with a bispecific antibody, the respective antibody was added to samples at a concentration of 10 µg/mL (in 10% FCS EMEM) and incubated at 37°C for 1 hr in order to allow sufficient time for antibodies to bind endogenous TGFβRII prior to addition of exogenous TGFβ.

Fibroblast-Derived Matrix Decellularization

Following 8 days of matrix deposition, matrices in 35-mm dishes were gently washed with PBS. 1 mL of pre-warmed extraction buffer (consisting of 0.5% Triton-X-100, 20 mM NH_4OH, and PBS + 1x protease inhibitors) was added to dishes and incubated for 5 min at 37°C or until confirmation of cell lysis. PBS with 1X protease inhibitors (2 mL) was added to the dishes containing extraction buffer and stored at 4°C overnight. The matrix was then treated with DNase I in PBS with Ca^{2+} and Mg^{2+} and incubated at 37°C for 30 min prior to staining.

Matrix Analysis by IF & SHG

Immunofluorescent (IF) images of phalloidin-stained samples were obtained with a Nikon Eclipse Ti-E inverted microscope at 10X magnification. Imaging of fibrillar collagen, fibronectin, and CHP in decellularized fibroblast-derived matrices was acquired by second harmonic generation using a Leica SP5 two-photon microscope (Leica Microsystems) at 10X magnification.

Flow Cytometry

Following 8 days of matrix deposition, fibroblasts were extracted and stained for FAP, CD90, and aSMA. Flow cytometry was performed on an LSR-Fortessa and data was analyzed using FlowJo.

Statistical Analysis

All quantitative analysis was performed using GraphPad PRISM 8.0 software (GraphPad Software, Inc.). Data was subsequently analyzed by one-way ANOVA with a Tukey's Test for multiple comparisons and shown as mean +/- standard error. Unless otherwise stated, a p-value < 0.05 was considered significant. Asterisks denote statistical significance: **** p < 0.0001, ***p < 0.001, ** p < 0.001, *p < 0.05.

Conclusion

- Cells in FDM-generating conditions can be driven to fibrogenic transitional myofibroblast phenotype by TGFβ, whereas cells on plastic exhibited high FAP+αSMAhi baseline levels and other stress response genes (data not shown).
- TGFβ drives a fibrogenic transitional myofibroblast phenotype – characterized by co-expression of fibroblast activation protein (FAP) and α-smooth muscle actin (αSMA) in fibroblast-derived matrix (FDM)-generating conditions.
- TGFβ induced increased cytoskeletal stress fiber formation and production of a more fibrotic-like matrix through the induction of fibrillogenesis and realignment of collagen fibers.
- Blocking TGFβ signaling with TGFβRII bispecific antibody only partially neutralized the TGFβ-driven phenotypic changes.