Myofibroblast Differentiation in Cardiomyocyte-Cardiac Fibroblast Bi-Layer Tissues on Tunable Synthetic Fibrous Matrices

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Introduction

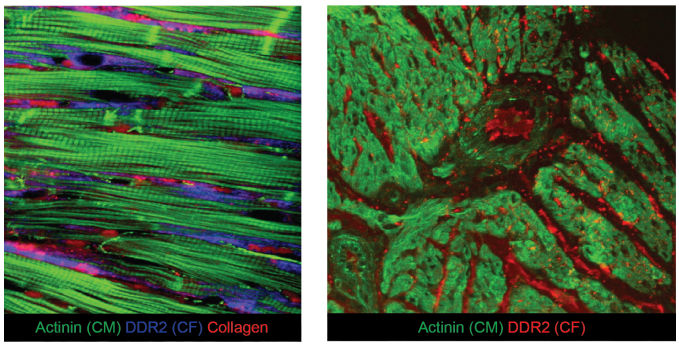


Figure 1. Fluorescent images of native heart tissue sections.[5, 6]

Cardiac pathologies are the leading cause of death in the United States[1] with cardiac fibrosis implicated in many of these diseases. A hallmark of cardiac fibrosis is the differentiation of cardiac fibroblasts (CFs) into myofibroblasts (MFs), which are responsible for depositing excessive amounts of extracellular matrix that mechanically impair heart function.[2] Previous studies have shown that modifying environmental mechanics[3] and communication between cardiomyocytes (CMs) and CFs[4] impact MF differentiation. However, the in vitro models used in these studies fail to accurately recapitulate the native architecture and organization of CMs and CFs present in the adult myocardium. In the adult myocardium, bundles of CMs are separated by collagen networks known as the endomysium (Fig. 1).[5, 6] CFs are interspersed within the endomysium, maintaining the collagen networks while signaling through the matrix to neighboring CMs.[5, 6, 7] Traditional co-culture approaches and 3D-engineered constructs randomly intermix CFs and CMs and thus fail to recreate the physiologic organization of these distinct cell populations.[8, 9] Here, we developed a novel bi-layer tissue platform (BLT) consisting of layers of CFs and CMs separated by a tunable synthetic fibrous matrix to mimic the ECM mechanics of healthy or diseased tissue states. Using this platform, we sought to understand how the synergy between mechanical cues and heterotypic cell-cell communication maintains tissue homeostasis vs. drives cardiac fibrosis.

Methods

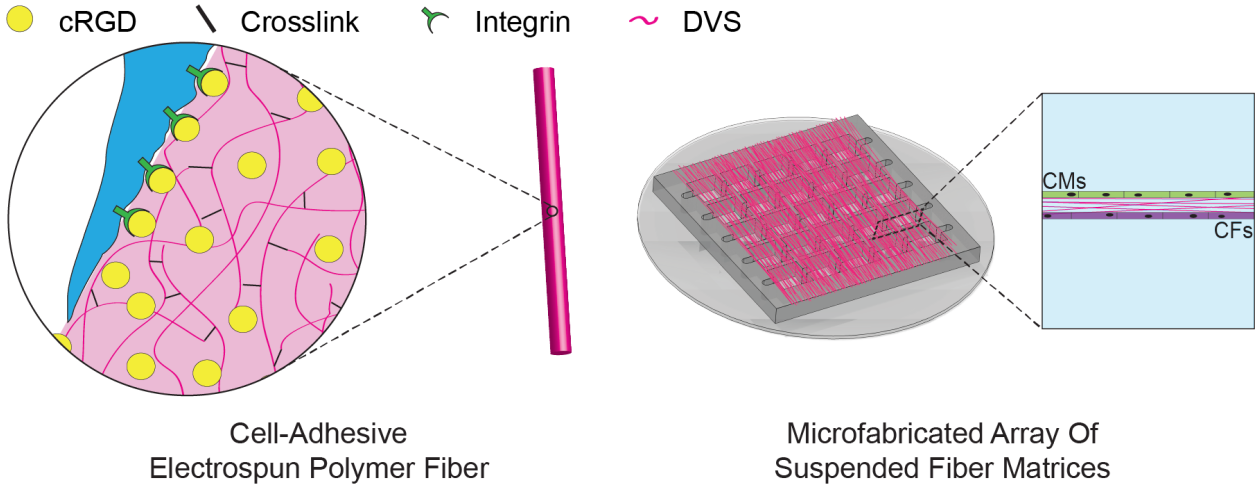


Figure 2. Schematic of the BLT. Monolayers of CMs and CFs are separated by an electrospun dextran vinyl sulfone (DVS) fiber matrix with tunable mechanical properties.

Substrate Fabrication with Tunable DVS Fiber Matrix

1. DVS fibers are electrospun onto microfabricated polydimethylsiloxane (PDMS) substrates containing an array of square microwells (2×2 mm).
2. Matrix alignment and stiffness were controlled by modulating the fiber collection mandrel speed and UV light exposure, respectively.
3. Fibers were functionalized with the cell-adhesive peptide RGD (CGRGDS).

BLT Formation and Culture

1. Induced pluripotent stem cell-derived CMs are seeded on top of the matrix and allowed to adhere for 3 days.
2. Normal human CFs (NHCFs) are pipetted down the channels of the substrate.
3. The substrate is inverted to allow the NHCFs to adhere to the bottom of the matrix.
4. The next day, the substrates are reoriented upright.
5. Cells are cultured for an additional 6 days in either RPMI B27 with insulin and Primocin or FGM3. Basal media is selectively supplemented with TGF-β1 and SB 431542.

Analysis of MF Differentiation

1. Immunostaining for α-smooth muscle actin (αSMA) is conducted to assess MF differentiation.
2. Samples are imaged using confocal microscopy and quantified using custom MATLAB scripts.

Results and Discussion

CFs spontaneously differentiate during expansion on tissue culture plastic

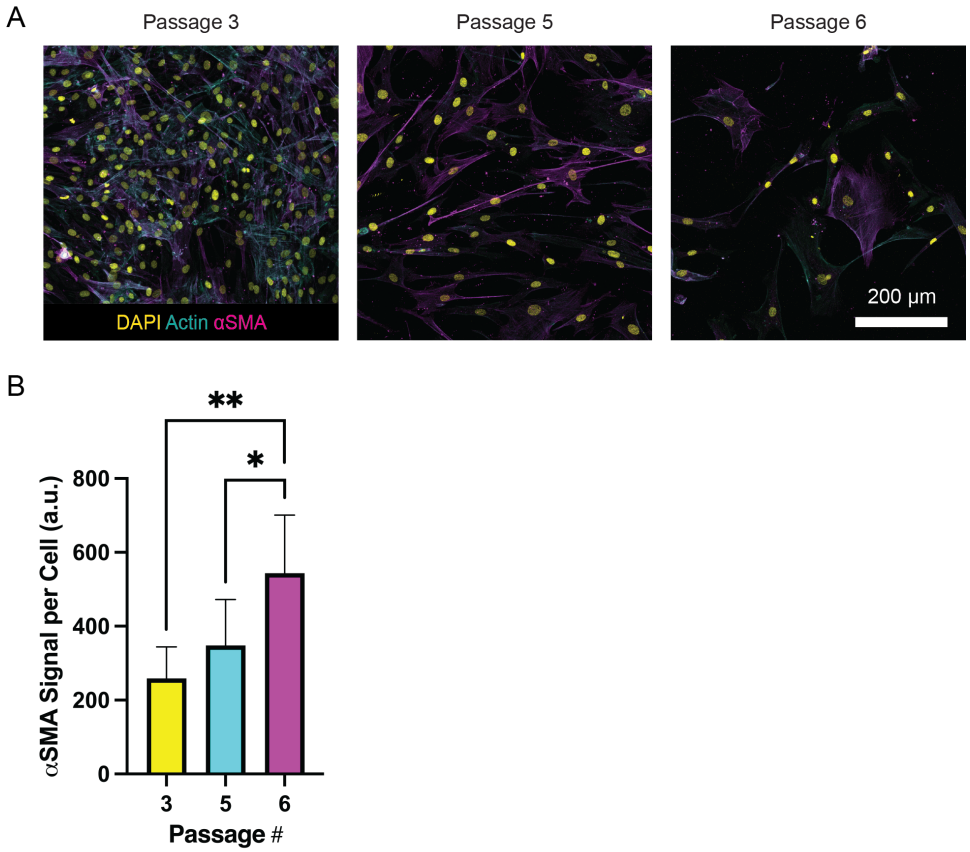


Figure 3. A) Representative fluorescent images of NHCFs cultured at various passage numbers on glass coverslips. B) Quantification of the αSMA signal per cell. * $p < 0.05$ and ** $p < 0.01$ by one-way ANOVA.

CF monocultures were cultured at various passage numbers on glass coverslips to characterize spontaneous MF differentiation during culture. The αSMA expression increases along with the passage number (Fig. 3B). The TGFβ receptor inhibitor SB 431542 will be added during NHCF expansion to mitigate MF differentiation.

Changes in ECM mechanics during cardiac fibrosis impact MF differentiation

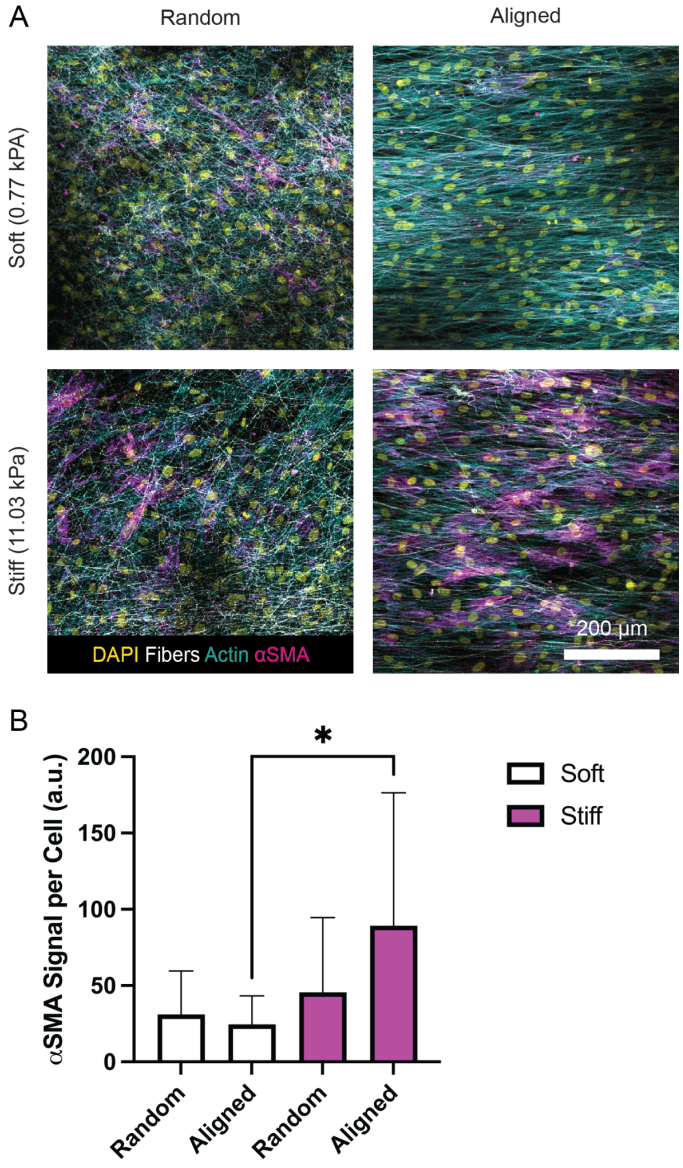
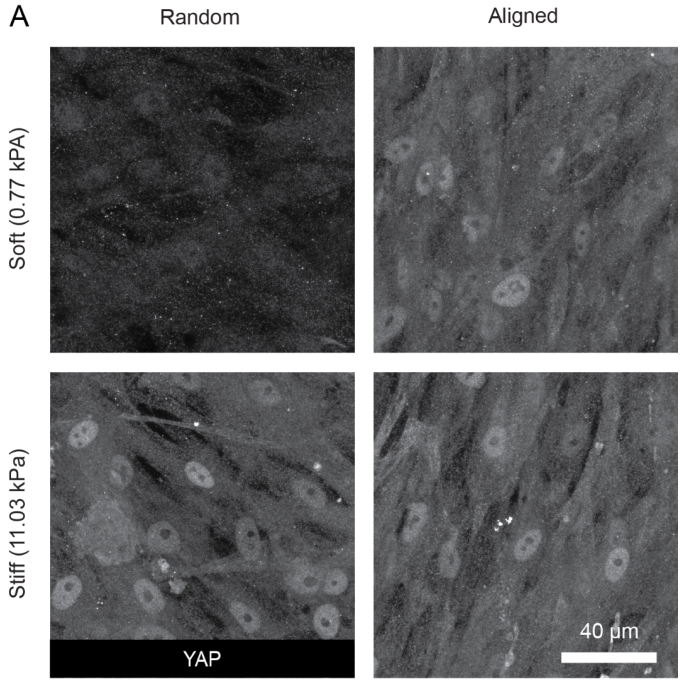


Figure 4. A) Representative fluorescent images of CF monocultures on varying matrix mechanics. B) Quantification of the αSMA signal per cell. * $p < 0.05$ by two-way ANOVA.

The effect of varying matrix mechanics on MF differentiation from CF monocultures was studied. Aligned, stiff (11.03 kPa) matrices induced significantly higher MF differentiation compared to random, soft (0.77 kPa); aligned, soft; and random, stiff matrices upon the addition of the pro-fibrotic cytokine TGF-β1 (Fig 4B). This supports the role of both soluble and mechanical cues in MF differentiation.

Changes in ECM mechanics during cardiac fibrosis impact cellular mechanotransduction



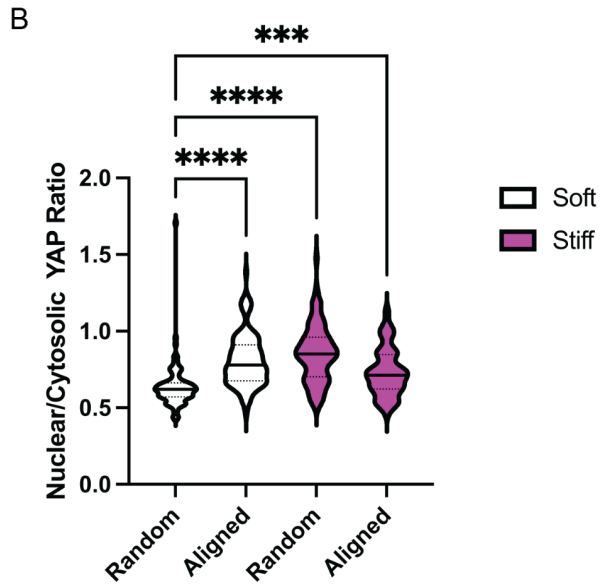


Figure 5. A) Representative fluorescent images of CF monocultures on varying matrix mechanics. B) Quantification of nuclear/cytosolic YAP localization. *** $p < 0.001$ and **** $p < 0.0001$ by two-way ANOVA.

The effect of varying matrix mechanics on CF mechanotransduction was studied. Nuclear localization of YAP was heightened in all conditions except for the soft, random matrices, further supporting altered mechanosensing in these environments (Fig. 5B).

Co-culture of CMs with CFs impacts MF differentiation

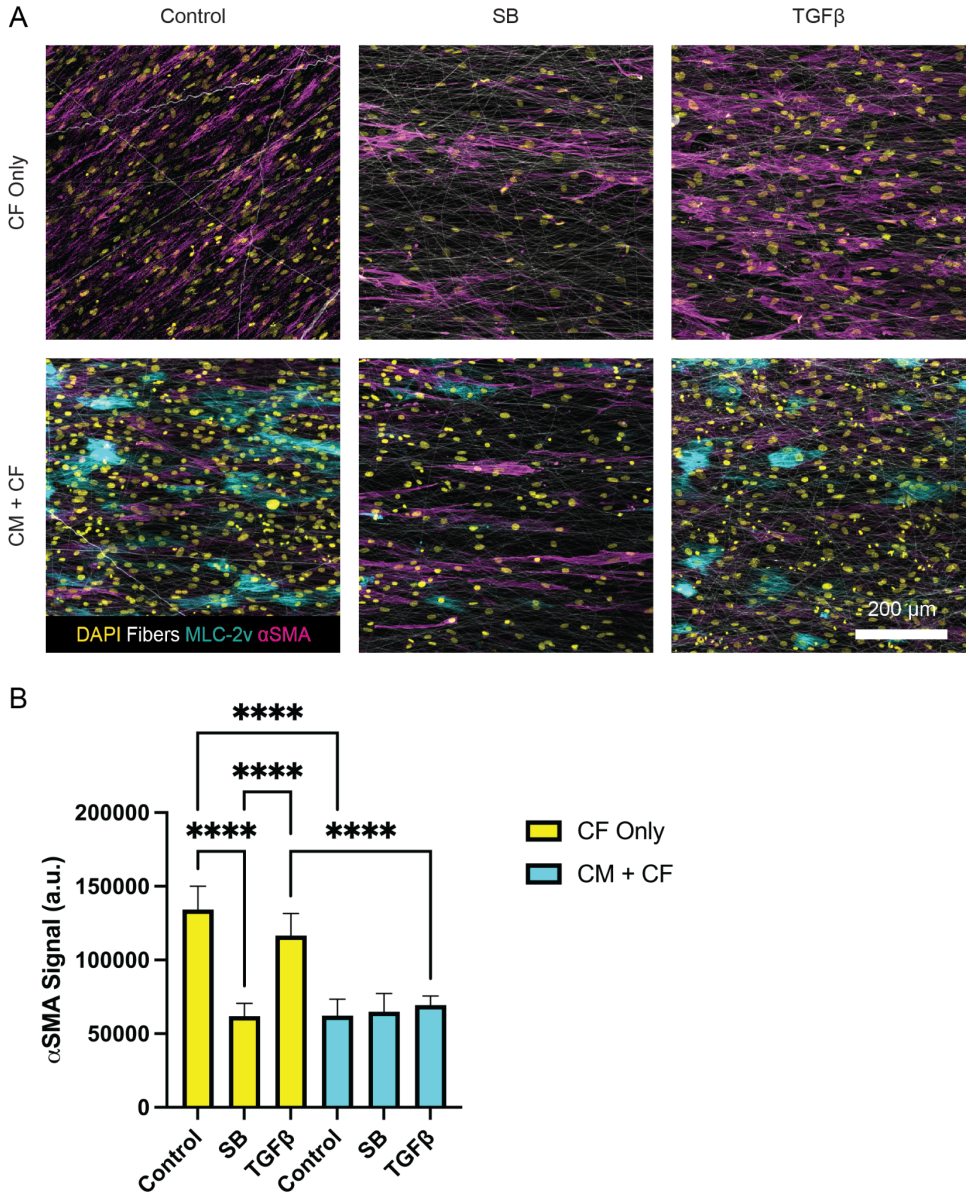


Figure 6. A) Representative fluorescent images of CF monocultures and CM + CF co-cultures. B) Quantification of the total αSMA signal. **** $p < 0.0001$ by two-way ANOVA.

Co-culture tissues were seeded to examine the influence of CMs on MF differentiation. Co-culture significantly decreased MF differentiation even in the presence of exogenous TGF-β1 (Fig. 6B). This suggests that protective signaling from the CMs impedes MF differentiation.

Orientation of BLTs does not impact the protective signaling from the CMs

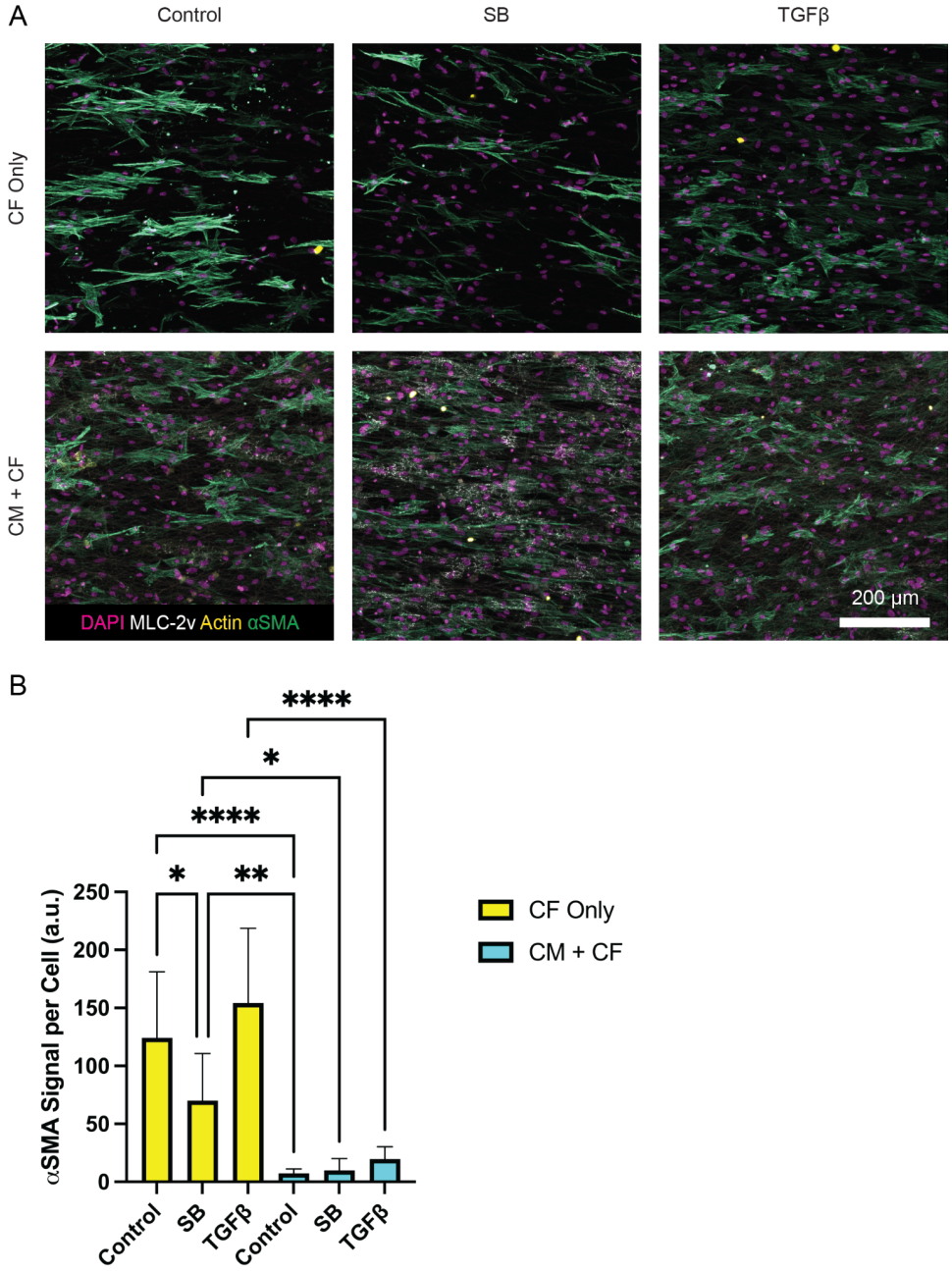


Figure 7. A) Representative fluorescent images of CF monocultures and CM + CF co-cultures. B) Quantification of the total αSMA signal. * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$ by two-way ANOVA.

Flipped co-culture tissues with the CMs on the bottom and CFs on top were seeded to examine the impact of flipping the orientation of the BLTs. The protective signaling from the CMs is retained (Fig. 7B).

Co-culture of CMs with CFs does not impact CM contractile behavior

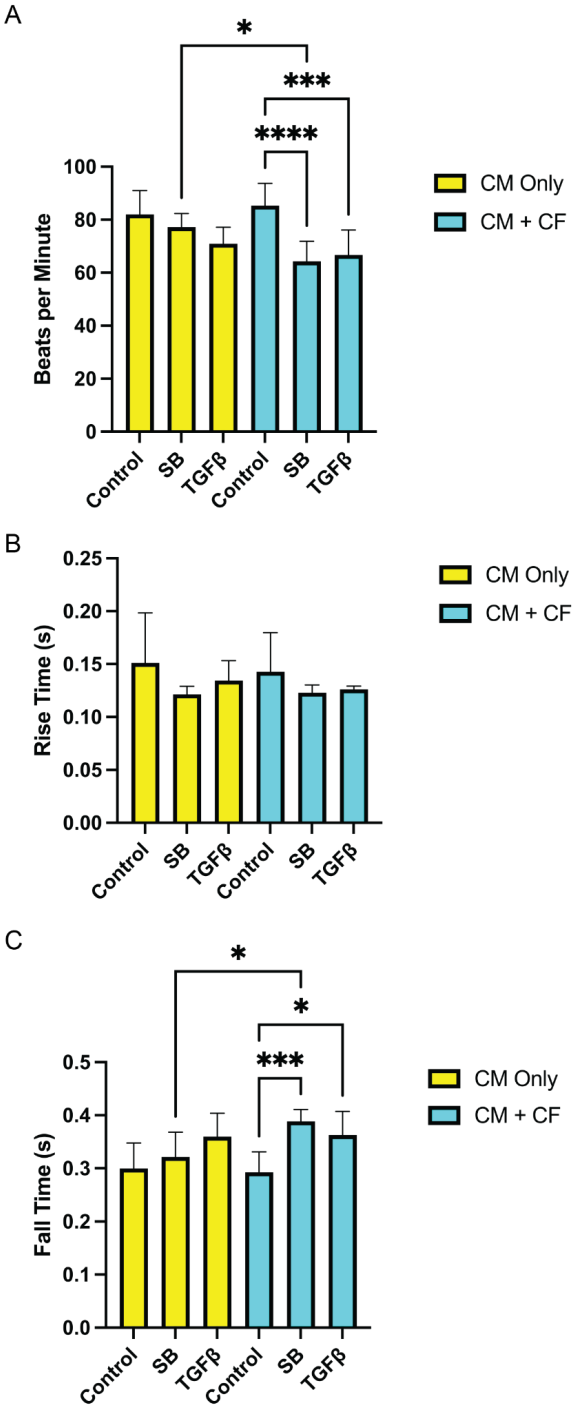


Figure 8. A) Quantification of the A) BPM, B) upstroke time, and C) downstroke time 80%. * $p < 0.05$, *** $p < 0.001$, and **** $p < 0.0001$ by two-way ANOVA.

Co-culture tissues were seeded to examine the influence of CFs on CM contractile behavior through calcium staining. The presence of CFs had little effect on CM contractions (Fig. 8A, 8B, 8C).

CM protective signaling requires cellular crosstalk

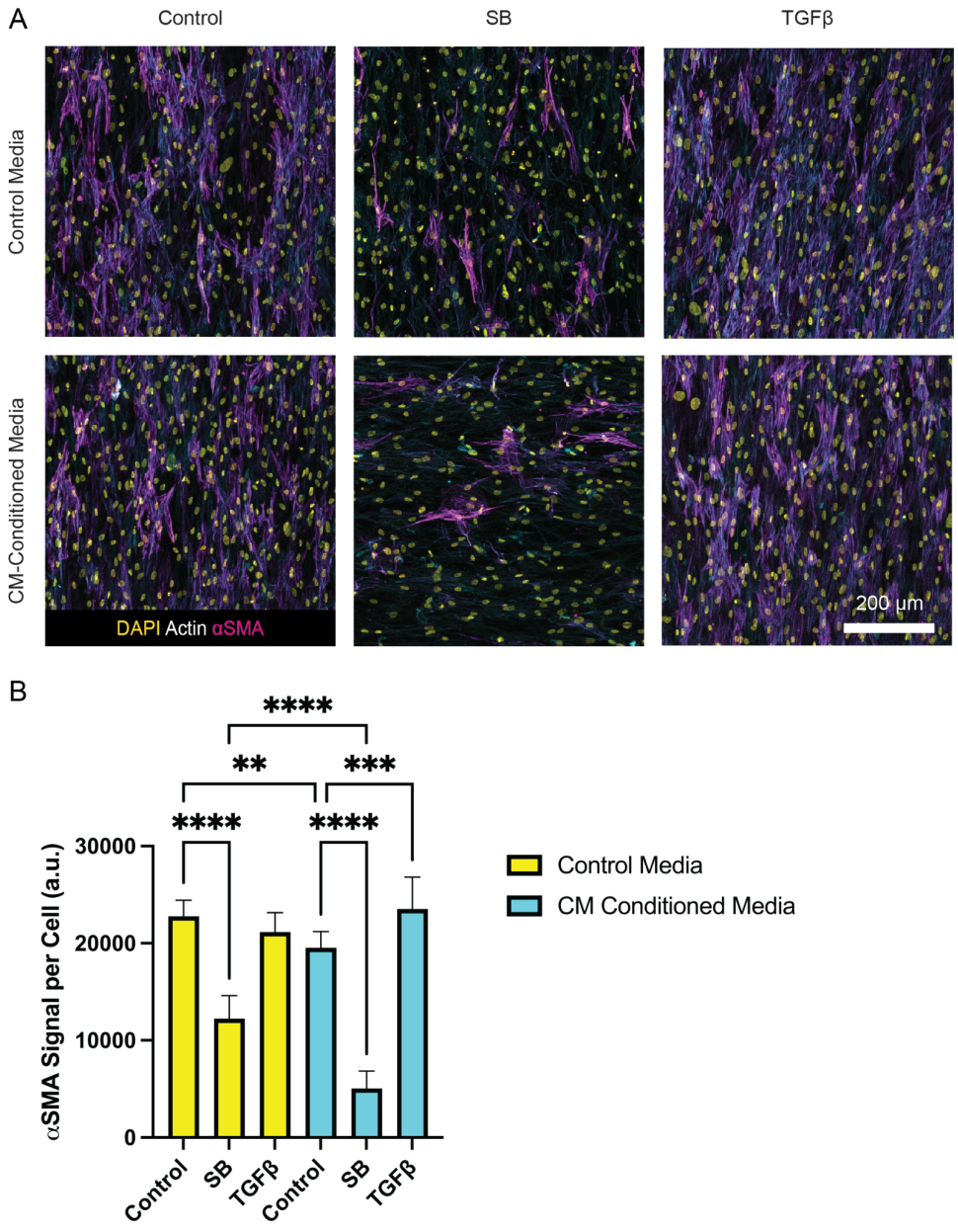


Figure 9. A) Representative fluorescent images of CF monocultures with either control or CM-conditioned media. B) Quantification of the αSMA signal per cell. ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by two-way ANOVA.

CF monocultures were cultured in either control or CM-conditioned media to characterize the protective effect of co-culture of CMs and CFs. Culture in CM-conditioned media led to a small

reduction in MF differentiation; however, this effect was negated by the presence of exogenous TGF-β1 (Fig. 9B). This suggests that CMs need to be cultured together with CFs to retain their protective effects and that cellular cross-talk is required.

Soluble factors drive CM protective signaling

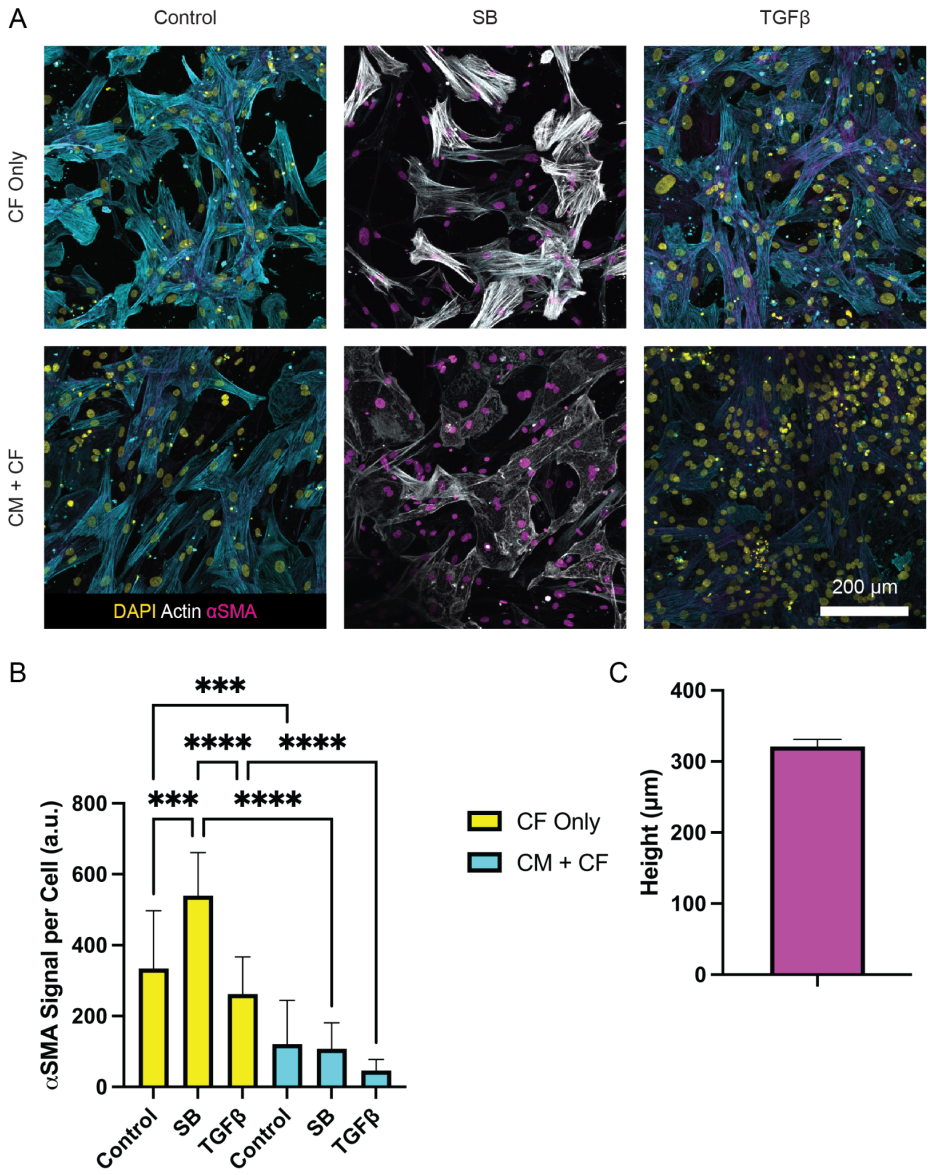


Figure 10. A) Representative fluorescent images of CFs ~300 μm away from CMs. B) Quantification of the αSMA signal per cell. C) Quantification of the distance between CMs and CFs. *** $p < 0.01$ and **** $p < 0.0001$ by two-way ANOVA.

CFs were seeded on the PDMS ~300 μm below the CMs on the fibrous matrix (Fig. 10C) to characterize the protective effect of CM + CF co-culture. CMs still significantly decreased MF

differentiation even with separation (Fig. 10B). This suggests that soluble factors from CMs inhibit MF differentiation.

Conclusions and Future Directions

In this work, we developed a novel co-culture platform to explore how matrix mechanics and co-culture with CMs impact MF differentiation. When further characterizing MF differentiation, we find that CFs spontaneously differentiate in monoculture. Our findings demonstrate that physical cues from the ECM play a critical role in MF differentiation, as supported by altered cellular mechanosensing as a function of matrix architecture and mechanics. Interestingly, co-culture with CMs hindered MF differentiation through protective paracrine signaling that necessitates CMs in the vicinity of CFs, potentially due to required reciprocal heterocellular crosstalk. Future work will explore the influence of matrix mechanics on CM-CF communication and identify the mechanisms by which CMs inhibit MF differentiation via secretomics analyses and RNA sequencing.

Acknowledgements

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