Substrate stiffness regulates meniscal fibrochondrocyte mechanotransduction

Daphney R. Chery[1,2], Tonia K. Tsinmam[1,2], Edward D. Bonnevie[1,2], Robert L. Mauck[1,2]
[1]University of Pennsylvania, Philadelphia, PA, [2]Corporal Michael J Crescenz VA Medical Center, Philadelphia, PA.
Daphney.Chery@pennmedicine.upenn.edu

Disclosures: Bonnevie (2, Fidia USA, 3b OrthoGenRx), RL Mauck (8, JOR Spine)

Introduction: Meniscal fibrochondrocytes (MFCs) play a critical role in the physiology, pathology, and repair of the knee meniscus [1]. As the meniscus regulates load distribution in the knee, endogenous meniscus cells experience dynamic physical forces in response to activities of daily living [1]. At the same time, meniscus tissue undergoes marked transformations in local structure and mechanics (which defines the cellular microenvironment) during development and with the onset of degeneration [2,3]. Not only do MFCs continuously sense their immediate surroundings, this microenvironment also regulates how dynamic physical cues are transmitted to the cells from the macroscale to the microscale [4]. A number of in vitro studies have demonstrated that both passive cues (such as stiffness and topography) and dynamic cues (such as osmotic stress, compression, tension, and hydrostatic pressure) can direct cell mechano-response [5-7]. One of the earliest and most fundamental of mechano-transductive responses is the rapid influx of calcium, which in turn regulates multiple downstream signaling pathways [8]. It is not clear how passive cues in the microenvironment impact these mechano-transductive responses of MFCs. Therefore, the first objective of this study was to determine if substrate mechanics influence baseline intracellular calcium $[Ca^{2+}]_i$ signaling in MFCs. We hypothesized that the baseline $[Ca^{2+}]_i$ signaling dynamics of MFCs would be stiffness dependent, with an increase in $[Ca^{2+}]_i$ fluctuations as a function of substrate stiffness. Next, given that meniscus cells are often culture expanded for such in vitro studies, and this expansion on stiff substrates may alter or prime their subsequent response (a so called 'mechanical memory') [9,10], we further queried how these baseline signatures would change with culture expansion.

Methods: Substrate Preparation: Polyacrylamide (PA) hydrogels of 5 and 55kPa were prepared following established protocols [11]. Gels were coated with fibronectin prior to cell seeding. Cell Isolation: MFCs were isolated from the outer region of juvenile bovine menisci. Menisci were sectioned into 1 mm^3 cubes and cells were allowed to migrate out onto tissue culture plastic (TCP) for 1.5 weeks, followed by trypsinization and expansion to passage 2 in a basal medium containing 10% fetal bovine serum and 1% penicillin/streptomycin/fungizone (PSF). These *passaged* MFCs were then seeded onto glass or PA gels of 55 kPa and 5 kPa stiffness. In addition, *fresh* MFCs were seeded onto these same surfaces (without culture expansion) by allowing the cells to migrate directly out of the native tissue and onto the substrates. Immunofluorescence (IF): Following established protocols, cells were fixed in 4% paraformaldehyde and stained with DAPI, YAP, and phallodin to visualize the nucleus, YAP localization, and f-actin, respectively [12]. Nuclear/cytoplasmic YAP ratio and cell spread area were quantified using Image J, as in [12,13]. $[Ca^{2+}]_i$ signaling: For all groups, seeded cells were labelled with Cal-520™-AM in DMEM at 37°C for 1 hr [4]. Time series images were obtained on a confocal microscope (every 5 seconds for 12 mins) using a 10x water immersion lens. The percentage of responding cells ($R_{\%}$) and the number of peaks ($\#_{peaks}$) in the observation window were quantified using a custom MATLAB code [4]. Statistics: Groups were compared using one- and two-way ANOVA with Bonferroni's post hoc testing. Statistical significance was set at $p < 0.05$.

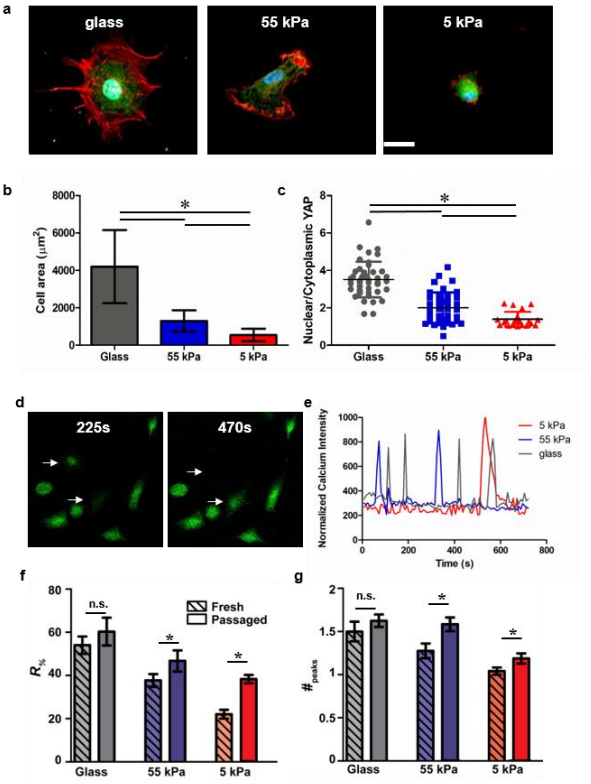

Figure 1. **a)** Representative IF images of MFCs on different substrates: glass, 55 kPa and 5 kPa, stained for YAP (green), F-actin (red) and nuclei (blue), scale bar = 25 μm. **b)** Quantification of cell spread area and **c)** nuclear/ cytoplasmic YAP ratio, error bars indicate S.D.; n > 30. **d)** Representative time series images of MFCs seeded on glass labeled with Cal-520™-AM. **e)** Typical calcium transients for a single MFC on each substrate. **f)** The percentage of responding cells ($R_{\%}$) and **g)** number of peaks ($\#_{peaks}$) for 'fresh' MFCs (that migrated directly onto substrates) compared with MFCs expanded to passage 2 (n > 40 cells per experimental group, errors bars indicate S.D.). Bars indicate S.D. * indicates $p < 0.05$.

Results: Our findings show that MFCs respond to variations in substrate stiffness (**Fig 1a**). MFCs on glass had a significantly greater cell spread area than on substrates with stiffnesses of 55 kPa and 5 kPa (**Fig 1b**). Consistent with other cell types [13], YAP was localized in the nucleus (activated) in MFCs on glass and 55 kPa, but was largely cytosolic (de-activated) on 5 kPa substrates (**Fig 1c**). When MFCs were loaded with a fluorescent $[Ca^{2+}]_i$ indicator, spontaneous $[Ca^{2+}]_i$ oscillations in single cells were recorded (**Fig 1d**), with representative transients on each of the three substrates depicted in **Fig 1e**. Quantification of this response indicated that increasing substrate stiffness enhanced $[Ca^{2+}]_i$ oscillations for *passaged* MFCs. Both the $R_{\%}$ and $\#_{peaks}$, increased as substrate stiffness increased (**Fig 1f,g**). Interestingly, when these same measures were taken for *fresh* MFCs that had migrated directly from the tissue onto the substrate, $R_{\%}$ and $\#_{peaks}$ was lower than in *passaged* cells on softer substrates (**Fig 1f,g**).

Discussion: In this study, we evaluated the mechano-responsiveness of MFCs on substrates of increasing stiffness. We found significant differences in MFC behavior on stiffer substrates, with increases in cell spread area, localization of YAP to the nucleus, and calcium signaling activity (**Fig 1a-g**). These data support that MFCs respond to such physical cues, consistent with other cell types [9,10,14], and further suggest that the developing and degenerative microenvironments of the meniscus may regulate cell activity. Moreover, our finding that culture expansion on a stiff substrate increased baseline MFC mechano-response (compared to those that had emerged directly from the tissue) indicates the possibility that MFCs may establish and retain a mechanical memory of their microenvironment (**Fig. 1f,g**), even after these conditions change. Future studies will investigate the mechanisms by which inputs from the generative or degenerative mechanical MFC microenvironment influence their future fate and function.

Significance: This study demonstrates that MFCs' mechano-responsiveness is stiffness dependent, and that mechanical priming can impact how these cells interpret changing environments. These data may inform and improve cell-based regenerative therapeutics for the knee meniscus.

References: [1] Makris, EA et al., *Biomaterials* 32:7411-7431, 2011. [2] Badlani, JT et al., *Am J Sports Med* 41:1238-1244, 2013. [3] Li, Q et al., *Acta Biomater* 54:356 366, 2017. [4] Han, WM et al., *Nat Mater* 15:477-484, 2016. [5] Fang, F et al., *J Orthop Res* 33:1704-1712, 2015. [6] Fang, F et al., *J Biomech* 47:2869-2877, 2014. [7] Upton, ML et al., *Biophys J* 95:2116-2124, 2008. [8] Guilak, F et al., *J Biomech* 47:1933-1940, 2014. [9] Godbout, C et al., *PLoS One* 8:e64560, 2013. [10] Yang, C et al., *Nat Mater* 13:645-652, 2014. [11] Aratyn-Schaus, Y et al., *J Vis Exp* 2010. [12] Driscoll, TP et al., *Biophys J* 108:2783-2793, 2015. [13] Cosgrove, BD et al., *Nat Mater* 15:1297-1306, 2016. [14] Heo, SJ et al., *J Orthop Res* 36:808-815, 2018.

Acknowledgements: This work was supported by the NIH (R01 AR075418, F32 AR072478), the NSF (CMMI-1548571), and a Penn Office of the Provost Postdoctoral Fellowship.