

## The Role of Cytoskeletal Adaptation in Mechanomics

Vina D. L. Putra<sup>1</sup>, Kristopher A. Kilian<sup>1</sup>, Melissa L. Knothe Tate<sup>2</sup>

<sup>1</sup>School of Chemistry and School of Materials Science & Engineering, University of New South Wales, NSW, Australia

<sup>2</sup>Blue Mountains World Interdisciplinary Innovation Institute (bmwi<sup>3</sup>), NSW, Australia  
proffate.bmwi3@gmail.com

**Disclosures:** Vina D. L. Putra (N), Kristopher A. Kilian (N), Melissa L. Knothe Tate (3C-TissuTex Pty. Ltd.)

**INTRODUCTION:** Stem cell *mechanomics* describes the effect of mechanical and biophysical cues on stem cell and matrix biology, eliciting the emergence of appropriate form for function [1,2]. Cellular mechanoadaptation underpins prenatal tissue development as well as postnatal tissue neogenesis and healing. In the current study we probed the role of cytoskeletal adaptation, *i.e.* actin and tubulin de-/polymerization and emergent cytoskeletal architectures, on cell shape, volume, and mechanical properties. We used increasing seeding density to introduce controlled compression to cells [3,4], substrates of increasing compliance to introduce controlled local tension to cells [5], and Paclitaxel (PAX), a microtubule depolymerization inhibitor, to control cytoskeletal dynamics [6].

**METHODS:** Cells of the C3H/10T1/2 murine embryonic stem cell line (CCL-226, a model mesenchymal stem cell MSC) were cultured at increasing density per previously published protocols [3,4] at 5000 cells/cm<sup>2</sup> for low density (LD), 15,000 cells/cm<sup>2</sup> for high density (HD) and 45,000 cells/cm<sup>2</sup> for very high density (VHD), and on substrates of increasing compliance [5]. In groups subjected to cytoskeletal stabilization, PAX treatment was carried out on the day following seeding, in culture medium at concentrations 1 – 100 nM. Cells were incubated until used for imaging or measurement at specific time points (24, 48, and 72h). For analysis of cytoskeletal structure, cells were fixed at pre-determined time points after PAX treatment with 4% paraformaldehyde for 10 minutes, permeabilized with 0.1% Triton™ X-100 in 1X PBS, and blocked with 10% FBS in PBS, with 3 times PBS wash in between. Cells were blocked for unspecific binding using 5% bovine serum albumin (BSA) in PBS for 1 hour. Then, tubulin was labelled with  $\alpha$ -tubulin monoclonal antibody 2  $\mu$ g/mL (Life Tech) for 3 hours at room temperature then crosslinked with secondary Goat anti-Mouse IgG conjugated with Alexa Fluor 568. For labelling actin, cells were stained with ActinGreen™ (AlexaFluor™ 488 phalloidin). Imaging was performed on Leica SP8 confocal microscope. Prior to imaging, cell nuclei were stained with Hoechst. Volume, surface area, and the surface area to volume ratio (SA/V) were quantified for cells and their nuclei. Statistical analysis was performed using Graphpad prism and SPSS. Significant differences in cell volume, stiffness, actin and microtubule concentration across PAX concentration, substrate stiffness and seeding densities were analyzed with Two-way ANOVA and Tukey's multiple comparison test. A linear mixed model was performed using the SPSS to investigate the effects of interacting variables on cells' Young's Modulus; (three-way interaction between independent variables: cell seeding density, substrate stiffness and PAX concentration) where data from each condition for each cell and repeat were pooled (results in **TABLE**). Bivariate Pearson's correlation analysis between the dependent variables such as cell volume, actin and microtubule concentration, actin alignment, and Young's Modulus, was performed to test how correlated their measures were from the three independent variables. The computed Pearson's correlation coefficient with two-tailed test of significance was used to define the negative, positive correlation or no correlation (results in **TABLE**). As not all combinations of variables were tested in this study, two-way ANOVA was also performed to test the interaction between two independent variables in modulating cell volume, actin and microtubule concentration, and actin alignment. Elastic moduli of cells were measured using Atomic Force Microscopy, using a JPK BioAFM by Bruker mounted on Nikon inverted microscope connected to stage heater set at 37°C within a TMC vibration isolation table. In addition, real-time deformability cytometry was carried out on suspended cells loaded into the syringe and flushed through the 30- $\mu$ m narrow channel constriction in a microfluidic chip (AcCellerator, Zellmechanik Dresden).

**RESULTS:** Data were compiled into a matrix (**TABLE**) demonstrating the correlation coefficient between all dependent variables measured in the study (pooled data) and indicating the level of interaction between cytoskeletal adaptation and/or remodeling parameters including cell volume (V), actin (ACT) alignment, and actin and microtubule (MT) concentration (conc.). In the Table, all significant ( $p < 0.05$ ) positive and negative correlations are shown. Values indicate Pearson's correlation coefficient. In context of this experimental model system where exposure to PAX is known to stabilize polymerized microtubules by inhibiting depolymerization, the 0.332 linear correlation coefficient measured between per cell microtubule concentration and PAX concentration can be taken as a reference point for "strongly correlated"; other correlation values can be considered relative to that known correlation, *i.e.* values above 0.3 were considered to exhibit strong positive correlation in this context.

**DISCUSSION:** Mechanical and biophysical cues intrinsic to increasing cell density (local compression) and substrate compliance (local tension) exert a greater effect than cytoskeletal stabilizing agents (PAX) in modulating MSC cytoskeletal adaptation. Furthermore, MSCs with stabilized microtubules (PAX treated) are sensitive to a range of substrate stiffnesses and seeding densities, indicative of a persistent albeit altered mechanoadaptation capacity of PAX treated cells and their nascent multicellular constructs.

**SIGNIFICANCE/CLINICAL RELEVANCE:** Elucidation of mechanisms by which MSC mechanoadaptation scales up across length and time scales is expected to provide innovative bottom-up design approaches for orthopaedic and regenerative medicine therapies and devices.

**REFERENCES:** [1] Anderson EJ and Knothe Tate ML (2007) *Tissue Eng* <http://doi.org/10.1089/ten.2006.0443> [2] Knothe Tate ML *et al.* (2016) *Bioarchitecture*, <https://doi.org/10.1080/19490992.2016.1229729> [3] McBride S and Knothe Tate ML (2008) *Tissue Eng Part A* <https://www.liebertpub.com/doi/10.1089/ten.tea.2008.0112> [4] Zimmerman J and Knothe Tate ML (2011) *Mol Cell Biomech* doi: 10.3970/mcb.2011.008.275 [5] Lee J *et al.* (2015) *Biomaterials* <https://www.sciencedirect.com/science/article/abs/pii/S0142961215006572> [6] Putra VDL *et al.* (2023) *Comm Biol* <https://www.scopus.com/record/display.uri?eid=2-s2.0-85146541024&origin=inward&txId=257295f87b778a9c90f768ecb5321785>

TABLE	PAX conc.	Compliance	Cell density	Cell V	ACT conc.	MT conc.	ACT align.	Modulus
PAX conc.	--	-0.144		0.341	0.328	0.332	0.287	0.263
Compliance	-0.144	--	0.215	0.404	0.174	0.171		-0.371
Cell density		0.215	--	0.285	-0.184	-0.111	-0.190	0.228
Cell V	0.341	0.404	0.285	--	0.244	0.416		
ACT conc.	0.328	0.174	-0.184	0.244	--	0.380	0.235	-0.116
MT conc.	0.332	0.171	-0.111	0.416	0.380	--	0.301	
ACT align.	0.287		-0.190		0.235	0.301	--	