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Extracellular matrix stiffness affects contractility in adult rat cardiomyocytes: implications for dynamic nitric oxide signalling and calcium handling

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Since techniques for cardiomyocyte (CM) isolation were first developed nearly four decades ago, experiments on single myocytes have yielded great insight into myocardial physiology. The stiffness of the myocardial extracellular matrix (ECM) is well known to be dynamic across age, with increases to modulus corresponding with age and pathology. The physiological effects of substrate modulus have been extensively investigated in neonatal *in vitro* CM models. However, our collective understanding of the effect of ECM stiffness on adult CM physiology and contractile function has been hampered by the difficulty of maintaining isolated adult CMs in culture. Adult CMs are notorious for low yield, high mortality and rapid de-differentiation after isolation. Furthermore, the ability to re-plate and examine a single cell over multiple conditions to examine post-isolation plasticity has not previously been realized.

In a recent article published in *The Journal of Physiology*, van Deel *et al.* (2017) introduce a model using isolated adult rat CMs that allows for the elucidation of modifications to contractility based on dynamic alterations to the substrate. Post-isolation, CMs were plated on surfaces of tunable stiffness, including those representing healthy (15 kPa) and diseased (100 kPa) myocardium. Contractile kinetics and Ca^{2+} flows were assessed immediately and at 24 h. Cells were also detached from their substrates via liberase digestion of their laminin anchors and re-plated on substrates of 15 or 100 kPa, before repeated measurement of the same physiological parameters. The authors noted a difference in contractility between cells plated on a 100 kPa surface (representing a diseased phenotype) for 24 h before subsequent re-plating on a 15 kPa (representing a healthy phenotype) *versus* a 100 kPa surface. To determine whether the observed changes in contractility were attributable to the microtubular organization, the authors visualized β-tubulin density and quantified α-tubulin acetylation as a measure of microtubule stability. However, neither analysis revealed significant differences between treatments, suggesting that microtubule dynamics were not responsible for the modulus-sensitive changes to contractility. Finally, the authors demonstrated the utility of their model by comparing the CM contractile parameters between lean ZSF1 rats and obese ZSF1 rats with heart failure with preserved ejection fraction (HFpEF); the latter phenotype is commonly used to model left ventricular stiffness and diastolic dysfunction. This experiment demonstrated phenotype-specific effects on calcium handling. Moreover, a significant difference in cell shortening and strong trends in contraction and relaxation velocity were observed between substrate stiffnesses.

In measuring Ca^{2+} handling and contractile properties, the authors revealed differential contractility between cells cultured on 100 kPa as opposed to 15 kPa substrates, both after re-plating from 100 kPa substrates. This reveals the robustness of these cells after three cumulative releases from ECM, which bodes well for further development of models of *in vitro* adult CMs for physiological investigation. More directly important, this reveals plasticity in some aspect of expression or organization of contractile machinery post-isolation, and hence the investigation into tubulin structure. As such, the likely candidate for these changes in contractility is still unknown.

This article adds to the field through the development of a variable-stiffness model with which to investigate mechanical contributions to adult CM physiology. Moreover, additional value is present in the physiology revealed by a relatively esoteric assay used in the article, showing the measurement of isometric force development using a permeabilized CM in an activation solution. This assay measures both the cell's maximal force development, mediated by the actin–myosin complex, and its passive tension (the force underlying a cell's return from a stretched to a relaxed state), controlled mainly by titin. The authors conclude that matrix stiffening induces an active response in the CM that leads to reduced cell shortening. Having previously found that differential regulation of titin contributes to myocardial stiffening in their HFpEF rat model, the authors suggest a similar mechanism as a contributing factor to the HFpEF phenotype, but not a primary factor to the change in contractility seen in HFpEF CMs. Finally, they suggest that the model introduced in this paper may be used to further investigate CM–ECM interactions, and encourage in-suspension measurement of contractile kinetics and Ca^{2+} handling in substrate-detached cells.

Titin, the giant protein most well known for its role in muscular passive stiffness, is likely not to be the main driver of changes to CM contractility observed in the *in vitro* experiments of this study. In addition to phosphorylation, the effects of titin can be regulated by isoform switching such as in dilated cardiomyopathy, which manifests in a decrease in passive myocardial tension (Nagueh, 2004). However, the permeabilized CMs used for force measurement in this study showed no changes to passive tension between treatments, lending little support to titin as an active mediator of the physiological changes observed. Moreover, as active contractile force was also not affected, it could be surmised that the cytoskeleton and associated contractile machinery are minimally affected by treatment.

By eliminating motor proteins or passive cytoskeletal proteins as mediators of changes to CM contractility, the authors conclude that the most likely candidates must therefore be involved in Ca^{2+} handling. All cell shortening and Ca^{2+} signalling was measured in cells freshly detached from the substrate. However, mechanical loading of CMs has previously been shown to induce nitric oxide (NO) signalling through induction of nitric oxide synthases (NOS) (Jian

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et al. 2014). The stiffness of the surface from which the cell is attached could ostensibly be proportional to the degree of impairment of post-detachment NO signalling. Certain NOS isoforms are known to co-localize with and increase the activity of the ryanodine receptor, increasing stiffness-induced contractile force (Jian *et al.* 2014). Given the rapid response of the pathway to changing conditions and the short half-life of NO (measured in seconds), detachment of the cell minutes before measurement could easily decrease activity of NO signalling and decrease Ca^{2+} transients and thus contractile force. A lower contractile force would in turn decrease both cell shortening velocity and magnitude, as seen in the current article. Furthermore, NO also induces sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity in a cGMP-independent pathway (Cohen & Adachi, 2006). Inhibited SERCA activity would impair Ca^{2+} reuptake and decrease shortening velocity proportionally to the stiffness of the membrane, as also seen in this article. In further support of this mechanism, Ca^{2+} amplitudes in cells cultured on stiff substrates, either singly or with replating, tended to be lower than in softer substrates. Finally, the authors found that the effects of substrate stiffness were further potentiated by the native myocardial stiffness of their HFpEF model. However, these cells could also be abrogating long-term, high-intensity NO signalling after detachment for cell shortening and Ca^{2+} handling measurements and thus demonstrating an enhanced version of the mechanism proposed above. These changes in NO signalling could be masking other physiological adaptations to the substrate undertaken over the 2-day culture period.

In a previous study (Galie *et al.* 2013), adult rat CMs plated on 255 kPa substrates demonstrated stiffness-specific transcription patterns of actinin, integrins and vinculin. These CMs demonstrated modest decreases in shortening and relaxation rates relative to those at lower stiffnesses more representative of nativ physiology, although not nearly to the degree of the CMs from stiff substrates in the study by van Deel *et al.* (2017). In the latter study, CMs plated on 100 kPa for 48 h and then detached tended to decrease by *ca* 25% in Ca^{2+} amplitudes; this was not mirrored when the CMs remained adhered to their substrate of similarly superphysiological stiffness for Ca^{2+} measurement (Galie *et al.* 2013). This discrepancy supports the hypothesis that rapid changes in NO signalling may be confounding the physiological changes observed.

One of the driving forces of this research was the development of a modality to assess CM contractility independent of passive inhibition by the surface to which the cells are currently attached. However, the model at hand is likely to induce unwanted physiological responses in the form of sensitive and rapid changes to NO signalling. Pharmacological inhibition of this pathway may be a method of overcoming these challenges, as may be genetic or siRNA workarounds. Regardless of these difficulties, with further development this research will certainly inspire improvements to the system in question and lead to greater physiological understanding. van Deel *et al.* (2017) have demonstrated robust, viable CMs that can endure significant handling, as well as a model of dynamic mechanical influence which may prove vital in further investigation of CM contractile physiology and pathology.

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Additional information

Competing interests

None reported.

Author contributions

Both authors contributed equally to the writing of the manuscript. Both authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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