Stem Cells Translational Medicine, 2022, **11**, 97–106
https://doi.org/10.1093/stcltm/szab002
Advance access publication 15 February 2022
Original Research

Stimulating Calcium Handling in hiPSC-Derived Engineered Cardiac Tissues Enhances Force Production

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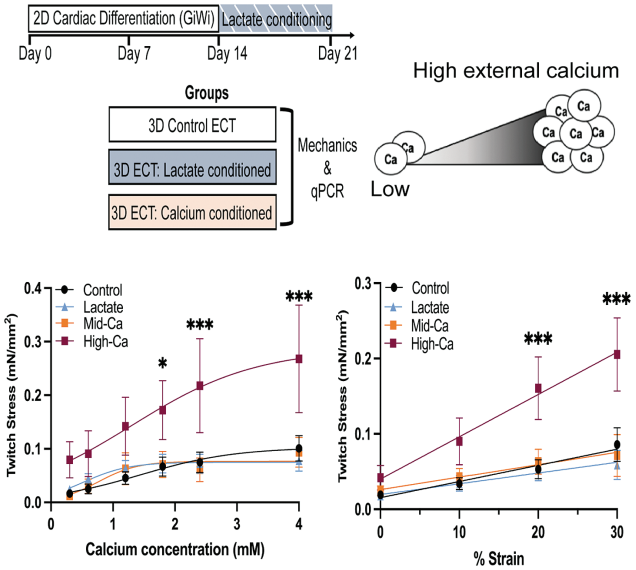
Abstract

Human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) have profound utility in generating functional human engineered cardiac tissues (ECT) for heart repair. However, the field at large is concerned about the relative immaturity of these hiPSC-CMs as we aim to develop clinically relevant models for regenerative therapy and drug testing. Herein, we develop a novel calcium (Ca^{2+}) conditioning protocol that maintains ECTs in a physiological range of Ca^{2+} and assesses contractility in increasing calcium environments. Lactate-based selection served as a method to purify and shift the metabolic profile of hiPSC-CMs to evaluate the role of metabolism on Ca^{2+} sensitivity. After 2 weeks, we observe 2-fold greater peak twitch stress in high-Ca^{2+} conditioned ECTs, despite having lower stiffness and no change in Ca^{2+} sensitivity of twitch force. Interestingly, the force-calcium relationship reveals higher Ca^{2+} sensitivity in lactate conditioned tissues, suggesting that metabolic maturation alters mitochondrial Ca^{2+} buffering and regulation. Ca^{2+} sensitivity and force amplitude are not coupled, as lactate conditioned tissues produce force comparable to that of controls in high calcium environments. An upregulation of calcium handling protein gene expression likely contributes to the greater Ca^{2+} sensitivity in lactate conditioned hiPSC-CMs. Our findings support the use of physiological Ca^{2+} to enhance the functional maturation of excitation-contraction coupling in hiPSC-CMs and demonstrate that metabolic changes induced by lactate conditioning alter cardiomyocyte sensitivity to external Ca^{2+}. These conditioning methods may be used to advance the development of engineered human cardiac tissue for translational applications in vitro and in vivo as a regenerative therapy.

Key words: calcium flux; human induced pluripotent stem cells (hiPSCs); cardiac; tissue engineering.

Graphical Abstract



Pre-conditioned engineered cardiac tissues formed from human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) demonstrate our ability to use a physiological calcium (Ca^{2+}) or lactate to increase contractility and sensitivity to external Ca^{2+}, respectively. We characterize the force-calcium relationship in hiPSC-CMs and define the role of Ca^{2+} and lactate as mediators to promote functional maturation of excitation-contraction coupling in hiPSC-CMs.

Received: 14 January 2021; Accepted: 6 September 2021.

Significance Statement

Current differentiation techniques have enabled production of human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes that present a fetal-like phenotype, driving interest in methods of maturation. Here, we condition engineered human cardiac tissue in physiological Ca^{2+} and report increased contractility. We also demonstrate an increased Ca^{2+} sensitivity of force with metabolic maturation through lactate conditioning with altered gene expression of Ca^{2+} handling machinery, implicating SERCA and mitochondrial Ca^{2+} buffering in modulating responsiveness to Ca^{2+}. Our novel approaches to maturing hiPSC-CM phenotype via enhanced calcium handling is a significant insight, useful for advancing regenerative therapeutics.

Introduction

Human-induced pluripotent stem cells (hiPSCs) are a clinically tractable cell source for regenerative cell therapy and disease modeling. Advancements in stem cell biology have enabled the directed differentiation of cardiomyocytes (CMs) from hiPSCs[1,2] and propelled the development of three-dimensional (3D) human engineered cardiac tissues (ECTs) for heart repair. As the field of cardiac tissue engineering continues to advance, greater attention has been given to assess the maturity of hiPSC-derived cardiomyocytes (hiPSC-CMs).[3,4] hiPSC-CMs often resemble fetal CMs in their gene expression, excitation-contraction (E-C) coupling, and contractility,[5] which makes it difficult to recapitulate the morphological and functional characteristics of adult heart tissue necessary for regenerating the myocardium and developing preclinical drug models. Recognizing these challenges, a number of approaches can be used to encourage maturation through mechanical stretching,[6,7] electrical stimulation,[8] and hormone treatment.[9]

E-C coupling is facilitated by the intracellular calcium (Ca^{2+}) transient, vital to the function of CMs. Mechanisms regulating E-C coupling have since been identified as targets to improve the maturation of hiPSC-CMs. Following an action potential, voltage-gated L-type Ca^{2+} channels ($Ca_v1.2$) transport Ca^{2+} across the cell membrane, activating Ca^{2+}-dependent ryanodine receptors (RyR2) on the sarcoplasmic reticulum (SR) to initiate calcium-induced calcium release (CICR).[10] This CICR causes a rapid increase in intracellular Ca^{2+} from ~100 nM to ~1 μM,[11] saturating troponin-C and facilitating actin-myosin binding for muscle contraction. Ca^{2+} is then pumped back into the SR via the Sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2), out of the cell via the Na^{+}-Ca^{2+} exchanger (NCX) or into mitochondria through mitochondrial calcium uniporter (MCU) channels.[10] MCU channels have a significant role in managing beat-to-beat oscillations of Ca^{2+} in neonatal cardiac myocytes[12] but the extent of mitochondrial Ca^{2+} buffering is reduced over the course of heart development. In adult CMs, mitochondria help maintain Ca^{2+} homeostasis, metabolism and E-C coupling.[13,14]

Proper calcium handling is crucial for the mechanical function of CMs but these calcium handling proteins are lowly expressed in hiPSC-CMs.[15] Several groups have shown that hiPSC-CMs and human embryonic stem cell-derived CMs (hESC-CMs) have a functional but immature SR, which has been implicated in poor functional integration and arrhythmia in vivo.[16] Differences in protein expression and organization contribute to immature calcium handling and inefficient E-C coupling in hiPSC-CMs compared to native adult ventricular CMs.[15,17] Ultrastructural differences in mitochondria and Ca^{2+} buffering capacity may also contribute to limited Ca^{2+} cycling and E-C metabolism coupling in hiPSC-CMs.[18] Therefore, in order to engineer functional, calcium responsive, 3D human cardiac tissues for heart repair we must determine ways to target, characterize, and improve E-C coupling in hiPSC-CMs.

In this study, we harness the crucial role of Ca^{2+} in E-C coupling to improve the structural and functional maturation of hiPSC-CMs. Because standard RPMI 1640 (Thermo Fisher Scientific) used for cardiac differentiation contains significantly less Ca^{2+} (0.42 mM) than the native myocardium, we developed a novel calcium conditioning protocol to maintain 3D engineered human cardiac tissue in medium (1.1 mM) and high (1.8 mM) concentrations of Ca^{2+}. Mid- and high concentrations of Ca^{2+} were selected based on the physiological range of Ca^{2+} in the heart (1.1-1.4 mM, ionized; 2.2-2.6 mM serum)[19] and compared to standard (low-Ca^{2+}) culture conditions. Further, because of the intimate relationships between mitochondria, metabolism, and E-C coupling, we used a common lactate selection protocol that purifies CMs from non-CM populations in hiPSC-CM cultures based on their metabolic use of lactate as a substrate[20] and drives metabolism to a more mature oxidative phosphorylative state.[21] We use lactate selection as a conditioning method to simulate the lactate-rich environment of the fetal heart, and by doing so, we were able to evaluate calcium handling properties of hiPSC-CMs, calcium conditioned CMs, and lactate conditioned CMs. Mechanical assessment, gene expression, and histological analysis revealed significant changes in cell phenotype indicative of shifts in Ca^{2+} sensitivity following high-Ca^{2+} and lactate conditioning. This work demonstrates our ability to specifically target and enhance the calcium handling properties of hiPSC-CMs and furthers the development of engineered human cardiac tissue as a clinical regenerative therapy and disease modeling.

Materials and Methods

Polydimethylsiloxane Tissue Mold Fabrication

Custom polydimethylsiloxane (PDMS) molds with troughs 3 mm wide and 17 mm long with posts (0.5 mm diameter) at both ends were fabricated to allow for engineered tissue formation, as previously described.[22,23] Briefly, negative acrylic master molds were designed in Adobe Illustrator then laser etched using an ULS 6.75 model laser cutter. PDMS was prepared according to the manufacturer's instructions and casted over the master molds. Molds were cured overnight at 60°C and autoclaved prior to use.

Cardiac Differentiation and Lactate Conditioning

hiPSCs (WTC GCaMP6 from The Gladstone Institutes; B. Conklin) were differentiated into CMs according to a well-adopted protocol using small molecule modulation of Wnt signaling (Fig. 1A).[2] Prior to differentiation, hiPSC colonies were maintained in chemically defined conditions on

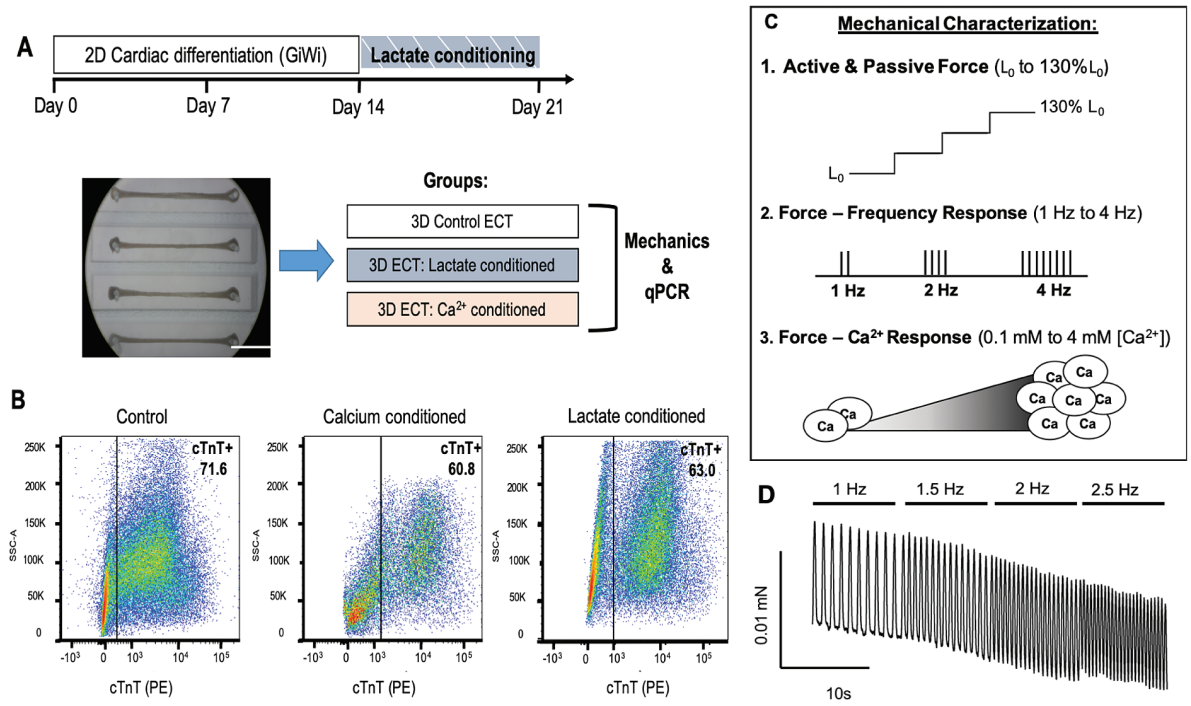


Figure 1. Schematic of cardiac differentiation and methods to characterize engineered cardiac tissue. (A) Cardiac differentiation was performed on 2D monolayers with certain groups undergoing lactate conditioning. Control, lactate, and calcium conditioned hiPSC-CMs were harvested to form 3D engineered cardiac tissues (ECTs) and maintained in test conditions for 2 weeks under continuous 1 Hz electrical stimulus. Scale bar = 5 mm. (B) Representative dot plots from flow cytometry used to determine cardiomyocyte purity. Gates for cTnT-positive populations were determined from compensation controls and unstained samples from each differentiation. (C) Sequence of mechanical characterization protocol used to evaluate active and passive force properties, force-frequency response, and force-calcium relationship of engineered cardiac tissue. (D) Representative force trace captured during electrical stimulation (1-2.5 Hz).

vitronectin (Thermo Fisher Scientific) coated tissue culture plastic dishes in Essential 8 Medium (E8, Thermo Fisher Scientific). Cell colonies were triturated into single cells and plated onto Matrigel (10 mg/mL, Corning) coated 24-well plates (Thermo Fisher Scientific) approximately 24 hours before cardiac differentiation.[21] To activate Wnt signaling, 6 µM CHIR 99021 (Chiron, Tocris), a glycogen synthase kinase 3 (GSK3) inhibitor, was delivered in cardiac differentiation media 3 (CDM3)[1] for 24 hours. Wnt signaling was deactivated 72 hours later using 5 µM Inhibitory Wnt Protein 2 (IWP2, Thermo Fisher Scientific) in CDM3. hiPSC-CMs typically obtained a beating phenotype after 10-12 days in culture, at which time they received RPMI 1640 medium with B27 supplement with insulin (RPMI+B27; Gibco).

hiPSC-CMs designated for lactate conditioning were harvested and replated at day 15 with RPMI+B27 (Fig. 1A). Media remained unchanged for 72 hours then washed with 1× phosphate-buffered saline (PBS) and fed lactate medium (8.3 g/L D5030 powder, 4 mM l-glutamine, 100× non-essential amino acids, 100× GlutaMAX, 4 mM lactate solution).[20] Lactate treatment was repeated after 48 hours then cells were allowed to recover for 2-3 days in RPMI+B27 medium prior to engineered tissue formation. Cardiac purity was assessed for all cell batches via flow cytometry using cardiac troponin T (cTnT) as a marker for CMs (Fig. 1B).

Preparation of Engineered Human Cardiac Tissues

PDMS molds were placed on the bottom of non-treated 6-well plates and treated with oxygen plasma to minimize electrostatic interactions between cells and the PDMS. ECTs were prepared by combining neutralized collagen I solution with hiPSC-CMs at a 1:1 ratio. On ice, rat tail collagen I solution (Advanced Biomatrix, San Diego, CA, USA) was neutralized as previously described.[23] hiPSC-CMs were suspended in RPMI+B27 then combined with neutralized collagen solution for a final density of 16×10^6 cells/mL in 1.2 mg/mL collagen. Tissues were cast by carefully dispensing 60 µL of cell and collagen mix into each trough of the tissue mold (3 × 17 mm). Following gelation at 37°C, engineered tissues received RPMI+B27 and 1% penicillin. After 48 hours, when cells had significantly compacted the collagen hydrogel, high-Ca^{2+} conditioned tissues received RPMI+B27 with 1.8 mM [Ca^{2+}], whereas control and lactate conditioned tissues received standard RPMI+B27 (0.42 mM [Ca^{2+}]). Mid-Ca^{2+} conditioned tissues received RPMI+B27 with 1.1 mM [Ca^{2+}]. All groups were supplemented with 1% penicillin, and media were exchanged every 48 hours. Tissues were cultured at 37°C and electrically paced under continuous 1 Hz stimulus for 2 weeks (C-Pace EP, IonOptix) (Fig. 1A). Brightfield optical microscopy images (Olympus SZ40) were taken for the first 5 days of culture to measure tissue compaction.

Mechanical Characterization of Engineered Human Cardiac Tissues

All experiments were performed using cell populations that had above 50% cardiac purity determined by cTnT expression as previous reports yielded optimal force production in the range of 40%-60% CM purity.[24,25] We used lactate and non-lactate conditioned cell batches with 50%-86% cardiac purity (Fig. 1B, Supplemental Table S1).

Active and passive force properties were analyzed for control, lactate, and calcium conditioned tissues 2 weeks after tissue formation (Fig. 1C). Tissues were carefully removed from PDMS molds, cut in half, and mounted via hooks onto a motor length controller and force transducer (5 mN load cell, Aurora Scientific) (Supplemental Fig. S1). Brightfield optical microscopy images (Olympus SZ40) were taken prior to testing to determine initial tissue dimensions at an initial length with no slack in the tissue. Active and passive mechanical testing were performed sequentially in a 37°C bath of Tyrode's solution containing: 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.4 mM KCl, 140 mM NaCl, 0.33 mM NaH_2PO_4, 10 mM HEPES, and 5 mM glucose at pH 7.4. Tissues were subsequently stretched to 130% of their initial length in 10% increments and held for 2 minutes at each strain to allow for stress relaxation. A 1 Hz electrical stimulus was delivered for 20 s to record active twitch contractions at each strain. Peak active stress was determined at 30% strain, normalized for cross-sectional area. Young's modulus (stiffness) was determined from the linear region (about 5%-20% strain, which is within the physiological strain regimen of the heart) of the passive force-length relationship. Following the 1 Hz stimulus at 30% strain, the force-frequency response was assessed by gradually increasing electrical stimulus to 4 Hz in 0.5 Hz increments (Fig. 1C, 1D). The maximum frequency at which the tissue could be paced is defined as the maximum capture rate (MCR).

Tissues were held at 30% strain for 4 minutes after the 4 Hz electrical stimulus to allow for equilibration in fresh Tyrode's solution containing 0.1 mM [Ca^{2+}]. However, force measurements show amplitudes higher than 0.3 mM [Ca^{2+}], giving us reason to believe that residual calcium was present from the previous 1.8 mM [Ca^{2+}] incubation, and therefore data from 0.1 mM [Ca^{2+}] were excluded from downstream analysis. The force-calcium response was analyzed by measuring twitch contractions for 20 s under 1 Hz stimulus in increasing extracellular Ca^{2+} concentrations up to 4 mM [Ca^{2+}] (Fig. 1C). Tyrode's incubation solutions were carefully exchanged following each stimulation and tissues were equilibrated for 2 minutes prior to testing. The rate of force increase is reported as upstroke velocity, v_{up} (units in stress/time) and gives an indication of the rate of Ca^{2+} increase. Peak stress was determined at 4 mM [Ca^{2+}], normalized for cross-sectional area. Concentration-response curves were fit to a sigmoidal dose-response curve and analyzed for the half-maximum effective concentration ($logEC_{50}$) and Hill slope (n_H).

Immunohistochemistry

Following mechanical assessment, tissues were fixed in 4% paraformaldehyde in the dark for 10 minutes at room temperature, processed into frozen blocks with optimal cutting temperature (OCT) compound, and sectioned into 10 µm sections. Sections were blocked with 1.5% normal goat serum in ICC PBS for 1 hour then incubated in primary antibodies against RyR2 (1:200 Abcam, Cambridge, UK) or SERCA2 (1:250 Sigma, St. Louis, MO, USA) and cardiac troponin I (1:100 Abcam) overnight at 4°C. Sections were washed in PBS then incubated in corresponding fluorescent secondary antibodies for 1 hour at room temperature. For sections stained for RyR2 and α-actinin, antigen retrieval was conducted via proteinase K digest (10 µg/mL; Roche, Basel, Switzerland) for 12 minutes at 37°C. A primary antibody against α-actinin (1:500 Abcam) was incubated overnight at 4°C, then detected with a fluorescent secondary antibody. All sections were counterstained for cell nuclei with DAPI (4′,6-diamidino-2-phenylindole) and imaged on an inverted confocal microscope under ×60 magnification (Olympus FV3000).

RNA Extraction for Gene Expression Analysis

Total RNA was isolated from ECTs following mechanical assessment using ReliaPrep RNA Miniprep Systems, according to the manufacturer's protocols (Promega, Madison, WI, USA). RNA yield and purity were quantified using a full-spectrum, UV-Vis spectrophotometer (Thermo Fisher Scientific NanoDrop 1000). Reverse transcription reactions were performed in triplicate using random hexamers to synthesize ~150 ng complementary DNA (cDNA) per sample (Applied Biosystems, Foster City, CA, USA). TaqMan gene expression assays for voltage-gated L-type calcium channel ($Ca_v1.2$), ryanodine receptor 2 (RyR2), phospholamban (PLN), calsequestrin (CASQ2), and SERCA2 were procured to amplify gene targets during real-time quantitative polymerase chain reaction (RT-qPCR) for 40 cycles (ViiA 7 Real Time PCR System) (Supplemental Table S2). Results were normalized to 18S rRNA housekeeping gene (18S) according to the $2^{-\Delta\Delta C}{}_T$ method.[26]

Statistical Analysis

All statistical analyses were performed in Prism 8 (GraphPad Inc., San Diego, CA, USA). Unpaired Student's *t* tests were performed to compare the means of 2 groups. For more than 2 groups, one- or two-way analysis of variance (ANOVA) tests with appropriate multiple comparison tests were conducted. Flow cytometry data were analyzed using FlowJo software (BD, Ashland, OR, USA). All error bars represent SEM. Differences were considered statistically significant for P values < .05.

Results

Calcium Conditioning Increases Force Amplitude of hiPSC-CMs

One of the main objectives of this study was to evaluate the force-calcium relationship of ECTs following calcium or lactate conditioning. We evaluated tissues that compacted and remodeled their collagenous matrix to ~40% of their initial width and resumed beating after 2 days in culture under continuous 1 Hz electrical stimulus. We measured the active twitch force generated in increasing extracellular Ca^{2+} concentrations up to 4 mM [Ca^{2+}]. Our data show that significantly greater force per cross-sectional area (stress) is produced by high-Ca^{2+} conditioned tissues at Ca^{2+} concentrations near and above physiological levels (1.2-4 mM) (Fig. 2A). Peak twitch stress at 4 mM [Ca^{2+}] was greater than 2-fold higher in high-Ca^{2+} conditioned tissues (0.26 ± 0.10 mN/mm^2) compared to control and lactate conditioned tissues, suggesting that at 30% strain and high external Ca^{2+}, the quantity of calcium that these tissues can move around the cell is greater and reflects the coupling of calcium influx, CICR, and contraction amplitude by myofilaments. Previous reports have shown a decrease in myofilament lattice spacing during force development increases calcium sensitivity and further emphasizes the link between intracellular [Ca^{2+}], physical stretch, force

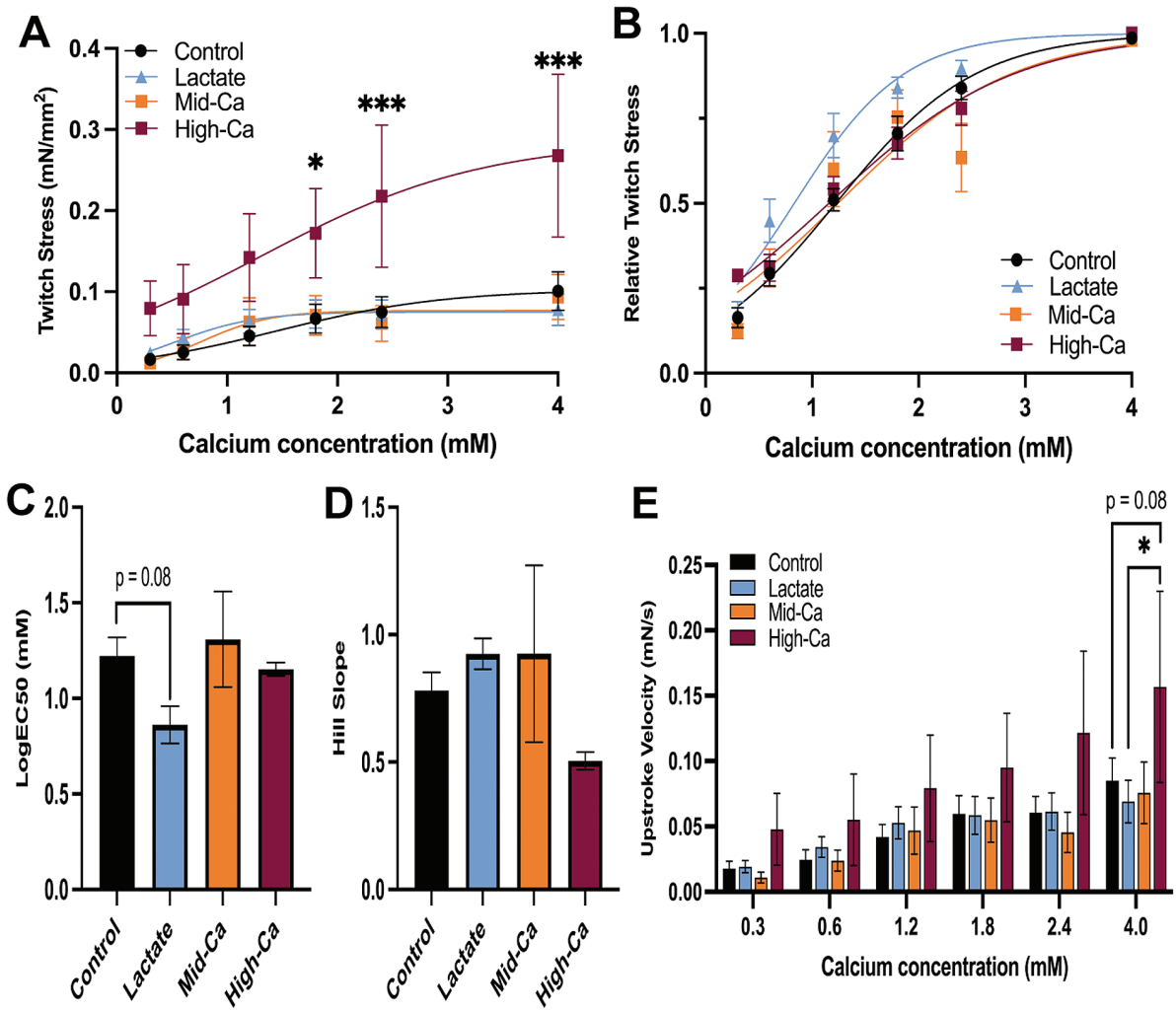


Figure 2. Force-calcium relationship of engineered human cardiac tissue. (A) Twitch force normalized by cross-sectional area (mN/mm^2) generated by engineered tissues in Tyrode's solution containing 0.3-4 mM [Ca^{2+}] after 2 weeks in culture. *$P < .05$ denotes significance between control and high-Ca^{2+} tissues, ***$P < .005$ denotes significance between high-Ca^{2+} and control, lactate, and mid-Ca^{2+} tissues. (B) Relative twitch stress as a function Ca^{2+} concentration normalized to maximum stress at 4 mM [Ca^{2+}]; nonlinear regression fit with sigmoidal dose-response curve shows shifts in calcium sensitivity. (C) $LogEC_{50}$ (mM) computed from nonlinear fit of each culture condition determines hiPSC-CM sensitivity to external Ca^{2+}. (D) Hill slope (n_H) determined from dose-response curves used as an index of cooperativity between Ca^{2+} binding and contractility. (E) Upstroke velocity (mN/s) corresponds to the rate of Ca^{2+} increase as hiPSC-CMs are exposed higher levels of external Ca^{2+}. Error bars represent mean ± SEM; *$P < .05$. Control, $n = 16$; high-Ca^{2+}, $n = 3$; mid-Ca^{2+}, $n = 5$; lactate, $n = 12$.

production, and calcium sensitivity.[27] There was, however, no difference in peak twitch stress at 4 mM [Ca^{2+}] between lactate conditioned tissues (0.078 ± 0.019 mN/mm^2), mid-Ca^{2+} conditioned tissues (0.093 ± 0.028 mN/mm^2), and control tissues (0.10 ± 0.024 mN/mm^2). To evaluate Ca^{2+} sensitivity, these data were normalized to peak twitch stress and fitted with a sigmoidal dose-response curve, which clearly shows how the twitch stress shifts left with Ca^{2+} exposure in lactate conditioned tissues (Fig. 2B).

Lactate Conditioning Increases Ca^{2+} Sensitivity of hiPSC-CMs

We measured the $logEC_{50}$ and n_H to assess calcium sensitivity resulting from each conditioning protocol. The mean $logEC_{50}$ for lactate conditioned tissues (0.86 ± 0.09 mM) was less than control tissues (1.22 ± 0.09 mM, $P = .08$), which reflects an increase in sensitivity to Ca^{2+} in lactate conditioned tissues (Fig. 2C). We did not find a difference in $logEC_{50}$ between control and calcium conditioned tissues showing similar degrees of Ca^{2+} sensitivity between groups. The n_H was not altered by pre-conditioning (Fig. 2D) but the shift in $logEC_{50}$ in lactate conditioned tissues suggests the ability to maximize force response with limited external Ca^{2+}. Interestingly, Ca^{2+} sensitivity is uncoupled from force amplitude (Fig. 2A, 2B).

Lastly, we quantified v_{up} during twitch contractions in increasing external Ca^{2+}, as this metric reflects calcium release dynamics when the contribution of transmembrane Ca^{2+} flux is altered. High-Ca^{2+} conditioned tissues had significantly higher upstroke velocities at high calcium concentrations >2 mM [Ca^{2+}], which is higher than the conditioning concentration itself (1.8 mM [Ca^{2+}]), reaching a peak of 0.157 ± 0.073 mN/s compared to lactate conditioned tissues (0.069 ± 0.016 mN/s) (Fig. 2E). The calcium kinetics of high-Ca^{2+} conditioned tissues also surpassed those of control (0.085 ± 0.017 mN/s, $P = .08$) and mid-Ca^{2+} conditioned tissues (0.076 ± 0.024 mN/s, $P = .09$), suggesting a difference in the maturation of calcium handling capabilities at super-physiological external Ca^{2+}. The increased v_{up} and force amplitude with high-Ca^{2+}

conditioned tissues suggest some increased kinetics, but likely due specifically to the handling of the greater total Ca^{2+} load.

Structural Changes in Lactate Conditioned Tissues Reflect Greater Sensitivity to External Ca^{2+}

We used immunohistochemistry to examine protein content and localization to assess how these may contribute to the greater degree of calcium sensitivity in lactate conditioned tissues. We were particularly interested in the presence and localization of RyR2 and SERCA2, 2 proteins primarily responsible for CICR and sequestration, respectively, in ventricular CMs (Fig. 3). RyR2 appeared punctate with no apparent localization (eg, to *z*-discs and diads) between groups while SERCA2 was present throughout the cytosol.

Physiological Force-Length, Stiffness, and Excitability of Conditioned Tissues

Lactate and calcium conditioning induced maturation of passive and active force properties of ECTs at physiological external Ca^{2+} (Fig. 4). All tissues exhibited a near-linear relationship between peak twitch stress (force normalized by cross-sectional area) and length up to 30% strain (Fig. 4A), as observed in native and ECT.[28] Significantly greater peak twitch stress was generated by high-Ca^{2+} conditioned tissues at 30% strain (0.20 ± 0.048 mN/mm^2) compared to mid-Ca^{2+} conditioned (0.071 ± 0.028 mN/mm^2), lactate conditioned (0.059 ± 0.019 mN/mm^2), and control tissues (0.086 ± 0.022 mN/mm^2). These data confirm our previous finding that physiological calcium conditioning enables hiPSC-CMs to handle greater Ca^{2+} load and in turn yield greater force amplitudes while maintaining physiological responses, such as the active force-length response (Fig. 4A).

Interestingly, the passive tissue mechanical properties were altered with lactate conditioning. The Young's modulus (stiffness) of lactate conditioned tissues (3.44 ± 0.72 kPa) was significantly higher than high-Ca^{2+} conditioned tissues (1.24 ± 0.31 kPa) (Fig. 4B). There was no significant difference in stiffness between lactate conditioned, mid-Ca^{2+} conditioned (1.82 ± 0.19 kPa), and control tissues (1.99 ± 0.34 kPa, P = .09). Lactate conditioned tissues have a stiffness at the low end of the range of neonatal rat myocardial stiffness (4-11 kPa), which is below that of native adult rat myocardium (12-46 kPa).[29,30] The low Young's modulus of high-Ca^{2+} conditioned tissues, in addition to our sequential testing protocol (Fig. 1C and Methods), likely permitted tissue damage that led to failure in this group, resulting in a loss of replicates during the force-frequency response and force-calcium response (Supplemental Fig. S2).

To evaluate the excitability of these conditioned cardiac tissues, we measured the mean MCR for each group. We found similar abilities across groups for responding to rapid pacing

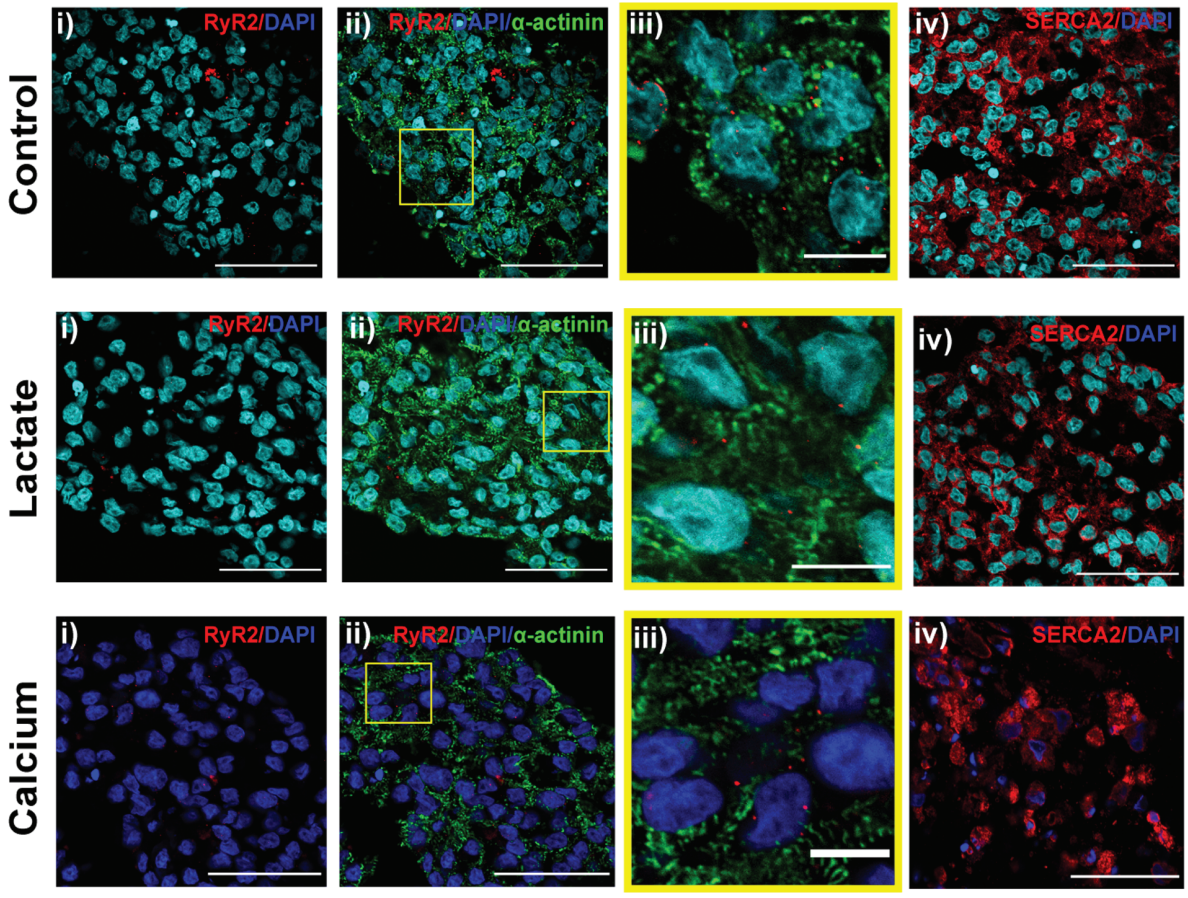


Figure 3. Histological analysis of pre-conditioned engineered cardiac tissue. Representative histological images of control, lactate, and calcium conditioned tissues stained for (i) RyR2 (red) and (ii) α-actinin (green) as a cardiac marker for myofilaments in hiPSC-CMs. Scale bar = 50 μm. (iii) Higher magnification of yellow boxed region in (ii) shows no apparent localization of punctate RyR2 protein. Scale bar = 10 μm. (iv) Sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2) (red) present throughout the cytoplasm of hiPSC-CMs with no specific localization. Scale bar = 50 μm.

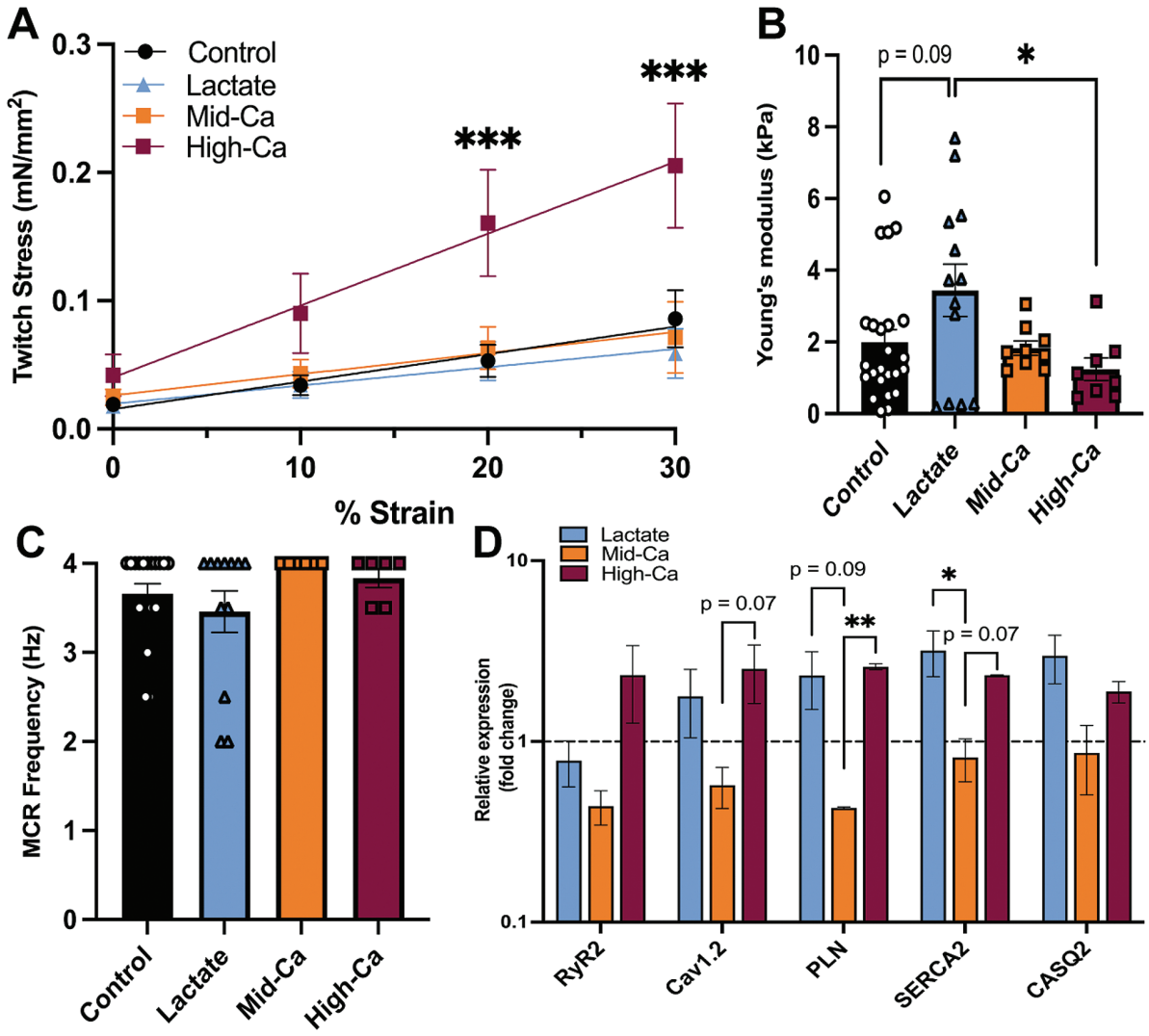


Figure 4. Functional assessment of engineered human cardiac tissue. (A) Peak twitch force normalized to cross-sectional area of tissue (stress) shows force-length relationship in 1.8 mM [Ca^{2+}] up to 30% strain. ***$P < .005$ denotes significance between high-Ca^{2+} and control, lactate, and mid-Ca^{2+} tissues. (B) Young's modulus (kPa) (stiffness) of tissues derived from passive force-length relationship. (C) Maximum capture rate (MCR) of tissues at 30% strain in 1.8 mM [Ca^{2+}]. Control, $n \geq 19$; lactate, $n \geq 12$; high-Ca^{2+}, $n \geq 6$; and mid-Ca^{2+}, $n \geq 6$. (D) Relative gene expression of calcium handling proteins at 2 weeks of culture. Normalized to 18S expression in each sample; fold change relative to control hiPSC-CMs (dotted line). Control, $n = 4$; lactate, $n = 5$; high-Ca^{2+}, $n = 2$; mid-Ca^{2+}, $n = 3$ from 3 differentiations. Error bars represent mean ± SEM; *$P < .05$, **$P < .01$.

rates, as tissues could be paced above a healthy adult human heart rate reaching mean MCR of 3.6 Hz, 3.8 Hz, 4.0 Hz, and 3.4 Hz for control, high-Ca^{2+}, mid-Ca^{2+}, and lactate conditioned tissues, respectively (Fig. 4C).

Pre-Conditioning Regulates the Expression of Calcium Handling Genes

Genotypic expression of proteins involved in calcium handling underlies the mechanisms of E-C coupling required to translate Ca^{2+} flux to force production. qPCR revealed no significant changes in RyR2 and CASQ2 gene expression in lactate or calcium conditioned tissues (Fig. 4D), suggesting little change in the calcium handling machinery responsible for the SR release and influx of Ca^{2+} into the cytosol and SR Ca^{2+} buffering. However, genes involved in calcium re-sequestration, SERCA2 and PLN, were upregulated following lactate conditioning and high-Ca^{2+} conditioning, respectively, compared to mid-Ca^{2+} tissues. The metabolic shift induced by lactate conditioning correlates with a greater expression of SERCA2 to regulate increased Ca^{2+} load and sensitivity. On the other hand, high-Ca^{2+} conditioning contributed to greater expression of PLN and slight increases in Cav1.2 ($P = .07$) and SERCA2 ($P = .07$) compared to mid-Ca^{2+} conditioned tissues, suggesting that high-Ca^{2+} but not mid-Ca^{2+} conditioning was sufficient to induce changes in gene expression after 2 weeks.

Discussion

hiPSC-CM maturity is an important consideration for developing in vitro models of the human myocardium and engineering regenerative therapies.[31-33] A hallmark of CM function is their ability to effectively translate calcium flux to contractile force, which is a novel target to induce hiPSC-CM maturation. In this study, we aimed to improve the calcium handling properties of hiPSC-CMs to induce more efficient E-C coupling toward a more mature CM phenotype. We took advantage of the vital role of Ca^{2+} in E-C coupling and

developed a novel calcium conditioning protocol that maintains hiPSC-CMs in chemically defined, physiologically relevant concentrations of 1.1 and 1.8 mM Ca^{2+} during engineered tissue culture to promote maturation of calcium handling. Further, with an understanding of the role of mitochondria on Ca^{2+} buffering in CMs[13] and how hiPSC-CM culture in a lactate-based medium matures metabolism toward oxidative phosphorylation,[21] we compared calcium conditioning to a widely used lactate conditioning/purification protocol[20] to assess the impact on calcium handling and contractility.

In 2017, the Zimmermann group reported that basal media for cardiac differentiation, RPMI 1640, contains 4-fold less Ca^{2+} (0.42 mM) than the native myocardium (1.8 mM), which may hinder the development of mature calcium handling.[24,34] We therefore hypothesized that conditioning engineered tissues with increased external Ca^{2+} or lactate as a metabolic substrate would increase Ca^{2+} sensitivity of force production with concomitant changes in gene expression. Indeed, our data demonstrate that maintaining hiPSC-CMs in high physiological Ca^{2+} (1.8 mM) increases the contractility of ECT ~2-fold after 2 weeks of culture compared to control tissues. All tissues were formed with similar CM purity (50%-86%) shown to promote good tissue formation[24,25] and cultured under 1 Hz electrical stimulus (Fig. 1B). High-Ca^{2+} conditions increased peak twitch stress by 2.5-fold to ~0.2 mN/mm^2 (Figs. 2A, 4A), thereby producing contractile strength about one-fifth of that of human infant heart tissue (~1 mN/mm^2).[35] In comparison to high-Ca^{2+} conditioned tissues, lactate conditioned tissues generated comparable amounts of force per cross-sectional area to control tissues, a result that parallels an earlier finding by our group[21] and suggests that lactate conditioning alone does not significantly increase contraction amplitude of hiPSC-CMs.

Analysis of passive force properties revealed a trade-off between tissue stiffness and active force production. As expected, all groups exhibited a linear force-length relationship to 130% of their initial length (Fig. 4A), consistent with the Frank-Starling Law of the heart.[28] However, lactate conditioned tissues were significantly stiffer than high-Ca^{2+} conditioned tissues (Fig. 4B). The stiffness of lactate conditioned tissues approaches that of fetal heart tissue, which enabled easier manipulation and mounting for mechanical testing. An earlier study from our group predicted that increasing tissue stiffness decreased active force production using a response surface methodology to evaluate the relationship between tissue stiffness, composition, and active force production.[30] In that study, as in the high-Ca^{2+} conditioned tissues here, tissues with the lowest stiffness and highest active force tended to fail by necking during mechanical testing. This may be explained by the disruption to intercellular junction formation caused by Ca^{2+}, which is necessary for tissue compaction and formation. In fact, during pilot studies, we learned that calcium conditioned tissues were unlikely to form a syncytium and uniformly beat in culture if surplus Ca^{2+} was present during tissue formation. We then amended our protocol to introduce physiological levels of Ca^{2+} after tissue compaction, but we speculate that a surplus of calcium ions in the culture environment may reduce tissue compaction and therefore stiffness even when adding Ca^{2+} after 2 days of tissue formation.

To assess E-C coupling with faster stimulation rates, we evaluated the MCR of ECTs in a force-frequency response and found that neither conditioning method altered MCR (Fig. 4C). All groups superseded the adult maximum heart rate of 2.5 Hz and up to 4 Hz. We did observe a negative force-frequency response (data not shown) similar to other cardiac tissue,[21,36] mimicking the flat or slightly decreasing trend found in newborn heart tissue.[35] If a force reduced force-frequency response is driven by slow relaxation, then no change in the force-frequency response would indicate that we have not changed the rate of Ca^{2+} re-sequestration despite the increase in SERCA2 and PLN protein (Fig. 4D). Additional electromechanical,[37] electrical,[38] and biochemical[39] stimuli may be incorporated in hiPSC-CM culture to promote a positive force-frequency response and further yield faster relaxation kinetics. Faster and complete relaxation following each twitch contraction may support higher force-frequency amplitudes, and future work could combine these conditioning regimens to promote a more physiological positive force-frequency response.

We were particularly interested in how lactate conditioning and calcium conditioning would affect force production as a function of increasing extracellular Ca^{2+}. High-Ca^{2+} conditioning increased twitch force amplitude in low and high calcium environments and ultimately produced a peak twitch stress 2-fold higher than control and lactate conditioned groups at high external Ca^{2+} (4 mM) (Fig. 2A). Similar studies with young hiPSC-CMs (1-2 weeks) have reported peak stress of 0.04-0.4 mN/mm^2 in high [Ca^{2+}].[24,40] Older hiPSC-CMs (4-8 weeks), however, generate peak stress between 0.4 and 6 mN/mm^2 in high external Ca^{2+}, demonstrating time-dependent variations in force across different systems.[8,24,40] As one may anticipate, pre-conditioning tissues in high physiological Ca^{2+} conditions for 2 weeks likely enabled cells to handle a greater load of Ca^{2+} from extracellular to intracellular SR stores and through CICR at any given pulse rate, which would be reflected in an increased contractile strength of calcium conditioned tissues at all extracellular Ca^{2+} levels due to higher availability of Ca^{2+} ions to bind myofilaments.

Interestingly, mid-Ca^{2+} and high-Ca^{2+} conditioned tissues did not exhibit similar behavior or functional maturity after 2 weeks. Later experiments revealed that ~75% of high-Ca^{2+} conditioned tissues lost their ability to beat spontaneously after 2 days in culture with 1.8 mM [Ca^{2+}] media while those in mid-Ca^{2+} conditioning media maintained their beating phenotype. We hypothesized that cells may have a graded response to external Ca^{2+} within a physiological range. Indeed, mid-Ca^{2+} conditioned tissues generated force not significantly different from control and lactate conditioned tissues (Figs. 2A, 4A) and had a gene expression profile closely resembling control tissues (Fig. 4D). This leads us to postulate that there is a threshold or range of external [Ca^{2+}] within 1.2-1.8 mM that facilitates functional and genotypic changes related to calcium handling in hiPSC-CMs and should be studied further.

Despite the significant increase in contractility in the high-Ca^{2+} conditioned group, lactate conditioning had the most profound effect on sensitivity to external Ca^{2+} (Fig. 2B). A lower $logEC_{50}$ for the lactate conditioned group (0.86 ± 0.09 mM) indicates that these tissues generated half their maximum force at a lower Ca^{2+} concentration compared to controls and are therefore more sensitive to Ca^{2+} (Fig. 2C). Our values are within the range of EC_{50} for ECT derived from other force-calcium analyses in literature (~0.5-1.8 mM).[24,36,40-42] We did not find a significant difference in $logEC_{50}$ between lactate conditioned and calcium conditioned groups. Further experiments indicated that cardiac purity had no effect on calcium sensitivity and support our hypothesis

that the observed changes in calcium sensitivity are the result of in vitro pre-conditioning (Supplemental Fig. S3).

The n_H provided an additional metric for sensitivity as an index of cooperativity between Ca^{2+} binding and contractility derived from the dose-response curves. Although we found no significant difference in n_H between groups (Fig. 2D), it is important to note that n_H and $logEC_{50}$ do not reveal molecular mechanisms, such as the phosphorylation of sarcomeric proteins, molecular arrangement of troponin, and myosin-actin binding affinity underlying cell sensitivity to calcium.[43] Further, the linear relationship between v_{up} and peak twitch stress was not changed by our pre-conditioning regimens (data not shown), suggesting that actomyosin cross-bridge kinetics were not affected by either lactate or calcium conditioning.

To further examine why high-Ca^{2+} conditioned tissues demonstrate greater peak active force, whereas lactate conditioned tissues express higher calcium sensitivity, we investigated structural features by immunohistochemistry. There was no apparent localization of SERCA2 and RyR2 in the cytosol of hiPSC-CMs suggesting the need to evaluate protein expression and assembly over longer culture periods (Fig. 3). Because of their greater passive tissue stiffness, lactate conditioned tissues may be more responsive to physical stretch. Therefore, at 30% strain, the stiffness of lactate conditioned tissues likely contributed to greater Ca^{2+} sensitivity. Gene expression data show an upregulation in SERCA2 and PLN following lactate conditioning and high-Ca^{2+} conditioning compared to mid-Ca^{2+} conditioned tissues (Fig. 4D). These proteins are typically lowly expressed in hiPSC-CMs and contribute to immature Ca^{2+} handling,[15,16] suggesting that changes in gene expression may occur through 2 weeks of in vitro conditioning to promote a more mature calcium handing phenotype.

This study demonstrates the importance of physiological Ca^{2+} to the structural and functional maturation of CMs derived from hiPSCs. Further, lactate conditioning induced an increase in calcium sensitivity of contraction, likely through its modulation of the tissue stiffness and metabolic phenotype that is a known buffer and modulator of Ca^{2+}. Thus, targeting the calcium handling properties of engineered human cardiac tissue is an effective strategy to yield greater sensitivity to Ca^{2+} and contractility via E-C coupling.

Conclusion

In summary, we developed a novel conditioning protocol using physiological levels of Ca^{2+} to improve the maturation and increase force production of hiPSC-CMs for cardiac tissue engineering. Further, assessment of an often-used lactate conditioning/purification protocol that drives metabolism toward oxidative phosphorylation also demonstrated increased Ca^{2+} sensitivity of force generation. These targeted approaches to enhance E-C coupling in hiPSC-CMs provide a better understanding of the role of Ca^{2+} and metabolism in the maturation of human ECT and advance the development of regenerative therapies for the myocardium.

Funding

This study was supported in part by grants from National Institutes of Health R01 HL135091 (to K.L.K.C.) with Diversity Supplement (to A.J.M.) and the Initiative to Maximize Student Development (IMSD) Training Grant (2R25GM083270-10 to A.J.M.).

Conflict of Interest

The authors declare no potential conflicts of interest.

Author Contributions

Conception and design, financial support, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript: A.J.M. Conception and design, financial support, administrative support, provision of study material, data interpretation, manuscript writing, final approval of manuscript: K.L.K.C.

Data Availability

The raw data from this study are available from the authors upon request.

Supplementary Material

Supplementary material is available at *Stem Cells Translational Medicine* online.

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