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Effects of Physiologic Mechanical Stimulation on Embryonic Chick Cardiomyocytes Using a Microfluidic Cardiac Cell Culture Model

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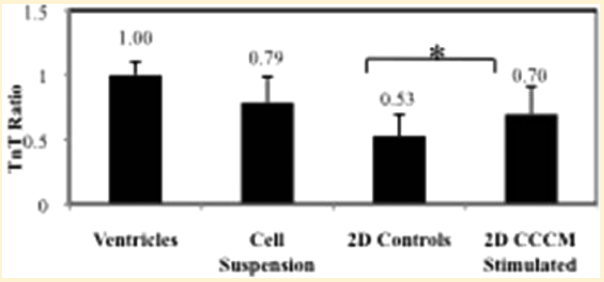
Supporting Information

ABSTRACT: Hemodynamic mechanical cues play a critical role in the early development and functional maturation of cardiomyocytes (CM). Therefore, tissue engineering approaches that incorporate immature CM into functional cardiac tissues capable of recovering or replacing damaged cardiac muscle require physiologically relevant environments to provide the appropriate mechanical cues. The goal of this work is to better understand the subcellular responses of immature cardiomyocytes using an *in vitro* cardiac cell culture model that realistically mimics *in vivo* mechanical conditions, including cyclical fluid flows, chamber pressures, and tissue strains that could be experienced by implanted cardiac tissues. Cardiomyocytes were cultured in a novel microfluidic cardiac cell culture model (CCCM) to achieve accurate replication of the mechanical cues experienced by ventricular CM. Day 10 chick embryonic ventricular CM (3.5×10^4 cell clusters per cell chamber) were cultured for 4 days in the CCCM under cyclic mechanical stimulation (10 mmHg, 8−15% stretch, 2 Hz frequency) and ventricular cells from the same embryo were cultured in a static condition for 4 days as controls. Additionally, ventricular cell suspensions and ventricular tissue from day 16 chick embryo were collected and analyzed for comparison with CCCM cultured CM. The gene expressions and protein synthesis of calcium handling proteins decreased significantly during the isolation process. Mechanical stimulation of the cultured CM using the CCCM resulted in an augmentation of gene expression and protein synthesis of calcium handling proteins compared to the 2D constructs cultured in the static conditions. Further, the CCCM conditioned 2D constructs have a higher beat rate and contractility response to isoproterenol. These results demonstrate that early mechanical stimulation of embryonic cardiac tissue is necessary for tissue proliferation and for protein synthesis of the calcium handling constituents required for tissue contractility. Thus, physiologic mechanical conditioning may be essential for generating functional cardiac patches for replacement of injured cardiac tissue.



Cardiovascular disease is the leading cause of morbidity and mortality in the world, resulting in a healthcare burden of >$300 billion annually. Cardiomyocyte apoptosis is the major cause of cardiac pathology that precedes heart failure. Cardiomyocytes stop differentiating and have limited proliferative potential after birth.[1,2] As a result, scar tissue replaces the damaged cardiac tissue, resulting in a loss of cardiac contractility and function. In addition, in vivo cardiac tissues are affected by the cyclic mechanical stimulation from hemodynamic loading and unloading (stretch, pressure, frequency, and fluid flow). These biomechanical cues are essential for the proper cardiac development and function,[3,4] and alterations in these biomechanical cues may result in cardiac dysfunction. A concerted effort is underway to restore cardiac tissue structure and function through regenerative approaches. Cardiac tissue engineering requires a suitable cell culture system for in vitro cardiac cell culture. However, replicating all aspects of the cyclic mechanical loading and unloading environment for the in vitro cardiac cultured cells is a challenging task as CM are under constant mechanical stimulation and rely on the conversion of these cues into intracellular signals to control cell phenotype and mass during growth and remodeling.

Our understanding of the molecular and cellular processes of in vitro cardiac tissue growth and remodeling is limited by the lack of a suitable culture system that replicates the

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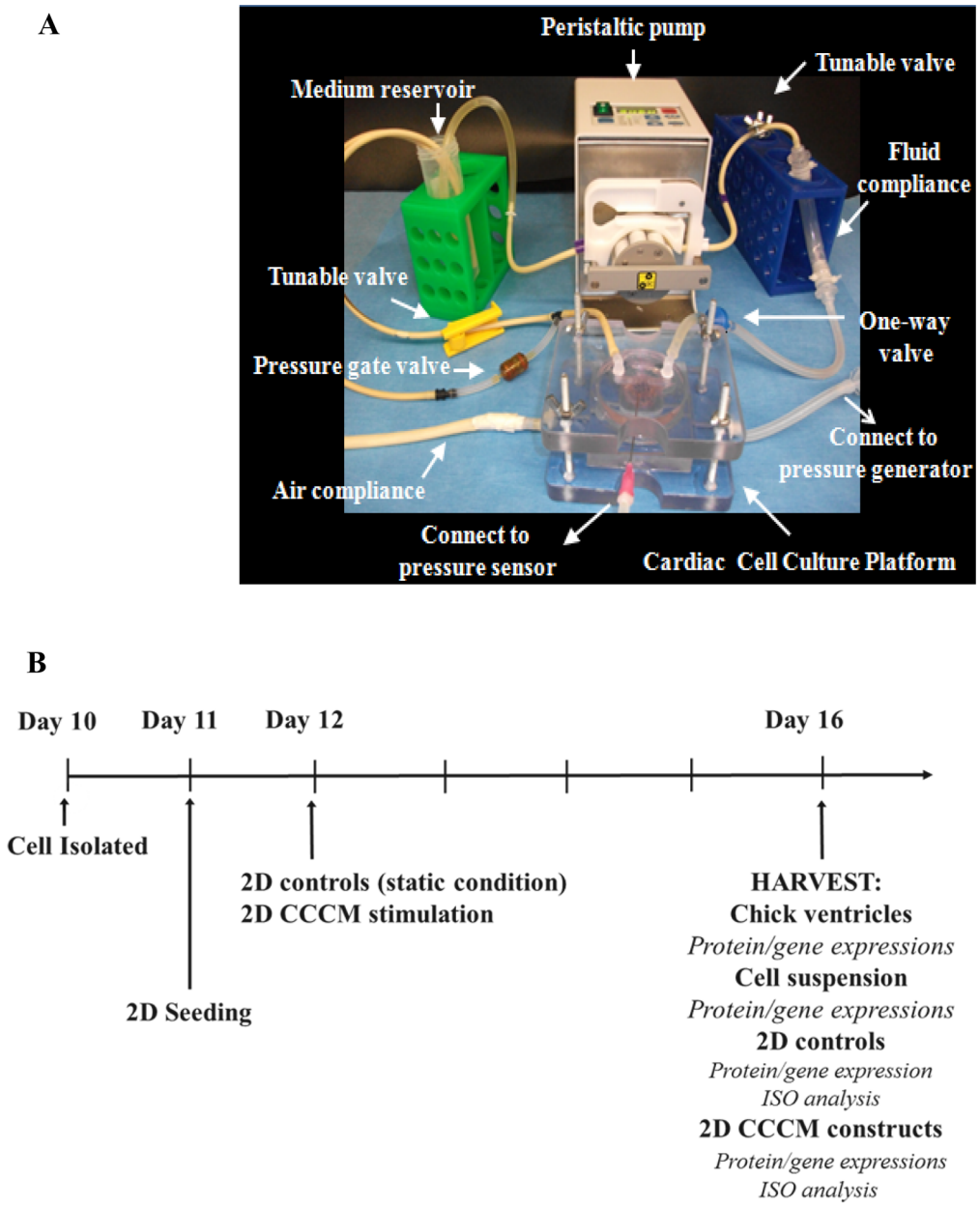

Figure 1. (A) Picture of the experimental setup of the CCCM system and (B) map of experimental design for ventricle chick embryonic cells. The day on the map was based on a 21 day chick embryo gestational.

biomechanical stimulation that occurs in vivo. We previously developed a dynamic and tunable cardiac cell culture model (CCCM) for cardiac tissue studies and generation (Figure 1A).[5] This system replicates all phases of the cardiac cycle and, thus, provides physiologic biomechanical cues necessary for cardiac tissue proliferation, differentiation, and functional maturation.

Subcellular responses of embryonic chick CM under physiologic mechanical stimulation were examined using the CCCM. Embryonic chick hearts have been used as primary cell lines for the study of cardiovascular functions and maturation.[6] In a previous study, embryonic chick CM cultured in CCCM demonstrated a higher proliferation rate, better alignment, higher contractility and beat rate (BR), and maturation.[5] We hypothesized that the higher contractility and BR may be due to augmented gene expression or proteins synthesis relating to the calcium handling regulation in response to physiologic mechanical stimulation in the CCCM. In this manuscript, the effect of mechanical stimulation on the gene expression and protein synthesis relating to calcium handling regulation proteins (sarcoplasmic reticulum calcium ATPase2a (SERCA2a), phospholamban (PLB), Troponin T (TnT), and Alpha and Beta-Myosin Heavy Chain (α-, β-MHC)) were evaluated. Further, the effects of the tissue exposure to isoproterenol (ISO) on β-adrenergic receptors (β-ARs) were also examined between the control and mechanically stimulated groups.

MATERIALS AND METHODS

Embryonic Chick Cardiomyocyte Isolation and Culture. Ventricular CM was isolated from fertile white leghorn chicken eggs (Charles River, North Franklin, CT), incubated in a forced-draft and constant-humidity incubator until day 10 (Hamburger-Hamilton stage 36). Excised day 10 embryonic left ventricles were digested with a 3 mg/mL collagenase type II

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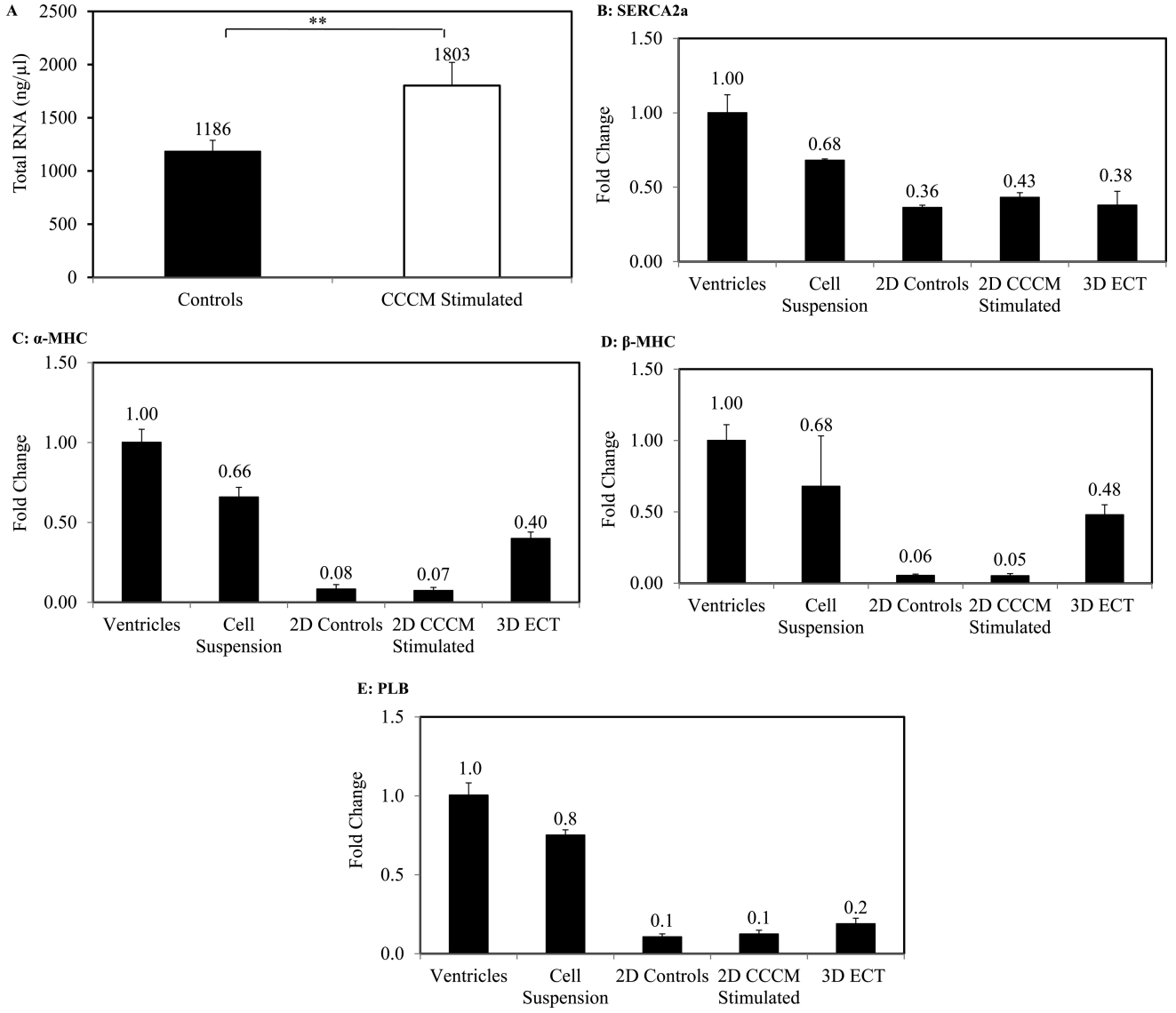

Figure 2. (A) Picture of the experimental setup of the CCCM system. Schematic of experimental design for chick ventricle embryonic cells. The day on the schematic was based on the 21-day chick embryo gestation. Abbreviation, ISO, isoproterenol. (B) SERCA 2a gene expressions, (C) α-MHC gene expressions, (D) β-MHC, and (E) PLB gene expressions. All samples were collected on day 16 (out of 21 days of gestation). Fold change for each gene of interest was determined by $\Delta\Delta C_t$ using chick ED16 ventricles as the calibrating sample for comparison to the native in vivo level of expression. $N \geq 3$ and $p > 0.05$. Abbreviations: ECT, engineered cardiac tissue; PLB, phospholamban.

solution, followed by a 0.05% trypsin-EDTA solution (Invitrogen, Carlsbad, CA) for 30 min. The CM were then filtered and preplated. After 1 h incubation, the cells were rotated overnight to reaggregate viable CM clusters.

Cardiomyocyte clusters ($\sim 3.5 \times 10^4$) were seeded on a glutaraldehyde cross-linked collagen matrix in each cell culture chamber. On day 2 of seeding, cells cultured in CCCM chambers were either conditioned by mechanical stimulations or cultured in a static condition as controls.

In order to compare gene expression between the 2D and 3D culture formats, isolated embryonic CM clusters ($\sim 3.0 \times 10^4$ clusters) were also mixed with collagen solution (0.667 mg/mL), seeded in the TT-4001C Tissue Train plates (Flexcell International Corporation, HillSborough, NC) using the Flexcell FX5K Tension system until the collagen solution cured to form 3D engineered cardiac tissues (ECTs) as previously published.[7,8] These ECT constructs were cultured under static conditions for 5 days prior to analysis for polymerase chain reaction (PCR) as described below.

Cell suspension from ventricular cardiac tissues from day 16 chick embryos were also isolated using a GentleMACST^M dissociator (MACS Miltenyi Biotec, Auburn, CA), preplated, and rotated before being collected for cDNA and proteins. At the same time, ventricular tissues from day 16 were also collected for cDNA and proteins. The overall experimental design is shown in Figure 1B.

Mechanical Stimulation within the CCCM. The cell culture chamber, with seeded cells, was transferred to the CCCM for mechanical stimulation for a period of 4 days at 37 °C and 5% CO_2. The fabrication, operation, CCCM preparation, and cell seeding methodologies have been previously published.[5] An 8−15% passive stretch and ∼10 mmHg peak pressure at a frequency of 2.0 Hz, with the fluid flow rate of 44 μL/cycle, were applied. At the end of 4 days of mechanical stimulation in the CCCM, the cell culture chamber

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was removed from the CCCM platform, cultured for 3 h in static condition, then analyzed as described below.

Isolation of RNA from Samples. Cardiac constructs were generated from at least three independent experiments. Fresh cell suspension samples, CCCM cardiac constructs, and chick embryonic ventricles were homogenized by an Omnitip tissue homogenizer (catalog no. 6615-7273, Scientific, Ocala, FL). Total RNA was isolated with an Invitrigen Trizol combined RNeasy MINI Kit (Qiagen, Valencia, CA; catalog no. 74104). RNA quality and quantity were measured using a NanoDrop ND-2000 (Thermo Fisher Scientific Inc., Waltham, MA). High-quality RNA were processed for qPCR.

Real Time Quantitative PCR. First strand cDNA synthesis was performed with a SuperScriptVILO cDNA Synthesis Kit (catalog no. 11754-050, Invitrogen) using 2.5 μg of RNA according to manufacturer's guidelines. Q-PCR was performed in triplicates using the TaqmanGene Expression Master Mix and TaqmanGene Expression Assay (SERCA2a, α and β MHC, PLB, and GAPDH), carried out on a ABI 7900HT system (Applied Biosystems, Foster City, CA). A fold change for each gene of interest was determined by $\Delta\Delta C_t$ using chick ED16 ventricles as the calibrating sample for comparison to the native in vivo level of expression.

Western Blotting. Total protein was collected from the samples using the Complete-Lysis M solution (Roche Diagnostics, Indianapolis, IN). Soluble protein concentration was determined using the NanoDrop 2000 Spectrophotometer. Total cellular proteins were separated using 8% and 10% SDS-PAGE and transferred to Immobilon-FL membranes (Millipore Corporation, Bedford, MA). After blocked with 5% nonfat milk, the membrane was probed with different primary antibodies including anti-SERCA2a (1:500) (LSBio, Seattle, WA), anti α-MHC (1:150) (Santa Cruz, Dallas, TX) and β-MHC (1:500) (Bioss, Woburn, MA), anti-Actinin (1:500) (Abcam, Cambridge, MA), anti TnT (1:1000) (Sigma-Aldrich, St. Louis, MO), and anti β-actin (1:1000) (Cell Signaling, Beverly, MA). The secondary antibody was Cy3goat anti-Rabbit IgG (1:500) (Life Technologies, Grand Island, NY) and Cy5 goat antimouse IgG1 (1:500) (Abcam, Cambridge, MA). The labeled membrane was finally visualized and photographed using a Typhon 9400 system and NIH ImageJ software. Protein content was compared between the CCCM control and CCCM stimulated groups. In contrast to the RNA analysis, we did not include parallel protein content from ED16 chick embryonic ventricles in this analysis.

Effect of Isoproterenol (ISO) on Stimulated Cardiac Tissue. The cell culture chamber, with the intact tissue construct, was put on a 37 °C thermo plate (Nikon) under a microscope. Increasing concentrations of ISO from 0.5 μM to 50 μM (Sigma-Aldrich, St. Louis, MO) were added to the cell chamber using a brief equilibration period after changes in ISO dose. For each ISO application, movies were recorded for beat rate and contractility analysis. Maximum CM contractility (% shortening) was calculated using a MATLAB program. Six experiments were performed.

Data Analysis. Data are reported as means ± SEM. At least three samples with paired data were used for data analysis in each experiment. Statistical significance was determined using two-tailed paired Student t test with significance assigned as $P < 0.05$.

RESULTS

Gene Expression. Figure 2A demonstrates total RNA synthesis obtained from 2D cardiac CCCM groups: the control and the stimulated ones. From the plot, the embryonic chick cardiac tissues stimulated yielded more RNA (1803 ± 218 ng/μL) than the tissues cultured under the static condition (1186 ± 102 ng/μL), which represents ~50% fold increase. Figure 2B–E demonstrates that gene expression decreased progressively from cells suspension to 2D CCCM culture compared to the level of gene expression in the embryonic chick ventricle for all three genes: SERCA2a, α-MHC, β-MHC, and PLB. The gene expression on 2D constructs, both the controls and the CCCM stimulated samples, was much lower than ventricular tissues or cell suspension on day 16, as was shown in a previous study.[9] Gene expressions between the control and the CCCM stimulated groups showed no significant change; however, expression of these genes was partially restored by 3D ECT in vitro culture.

Protein Synthesis. More than 10 paired samples (controls and CCCM stimulated groups) were used to determine total protein in each group. Total protein in the CCCD stimulated constructs significantly increased (97.15 ± 4.97 mg) versus total protein in 2D controls (71.52 ± 4.14 mg) (Figure 3)

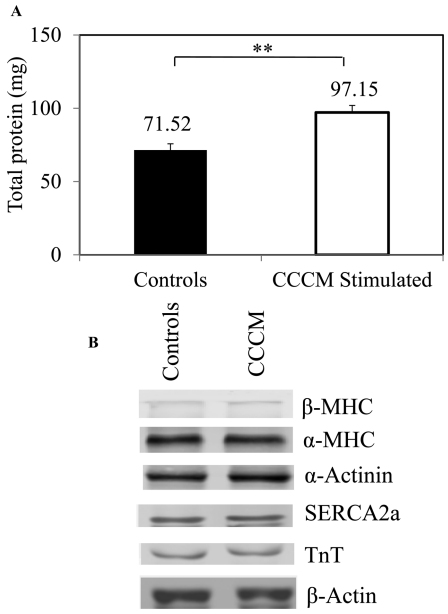

Figure 3. (A) Total proteins of the 2D cardiac tissue constructs. $N = 12$. Two tailed significance was set at ** $p < 0.005$. (B) Images of Western blot bands of the interested proteins in the 2D control and CCCM stimulated groups.

Five different CM proteins (SERCA2a, α- and β-MHC, α-actinin, and TnT) were selected for the Western blot (Figure 4). The target proteins collected from different conditions are shown in Figures 3 and 4. Proteins SERCA2a, TnT, and β-MHC from the cell suspension were much lower than those in the ventricular tissues. Interestingly, the amount of α-Actinin protein almost doubled after cell isolation from ventricular tissue. As noted in the previous study, protein levels decreased in response to 2D culture. However, when mechanically conditioned in the CCCM system, protein levels were modestly increased, except for myosin heavy chain proteins.

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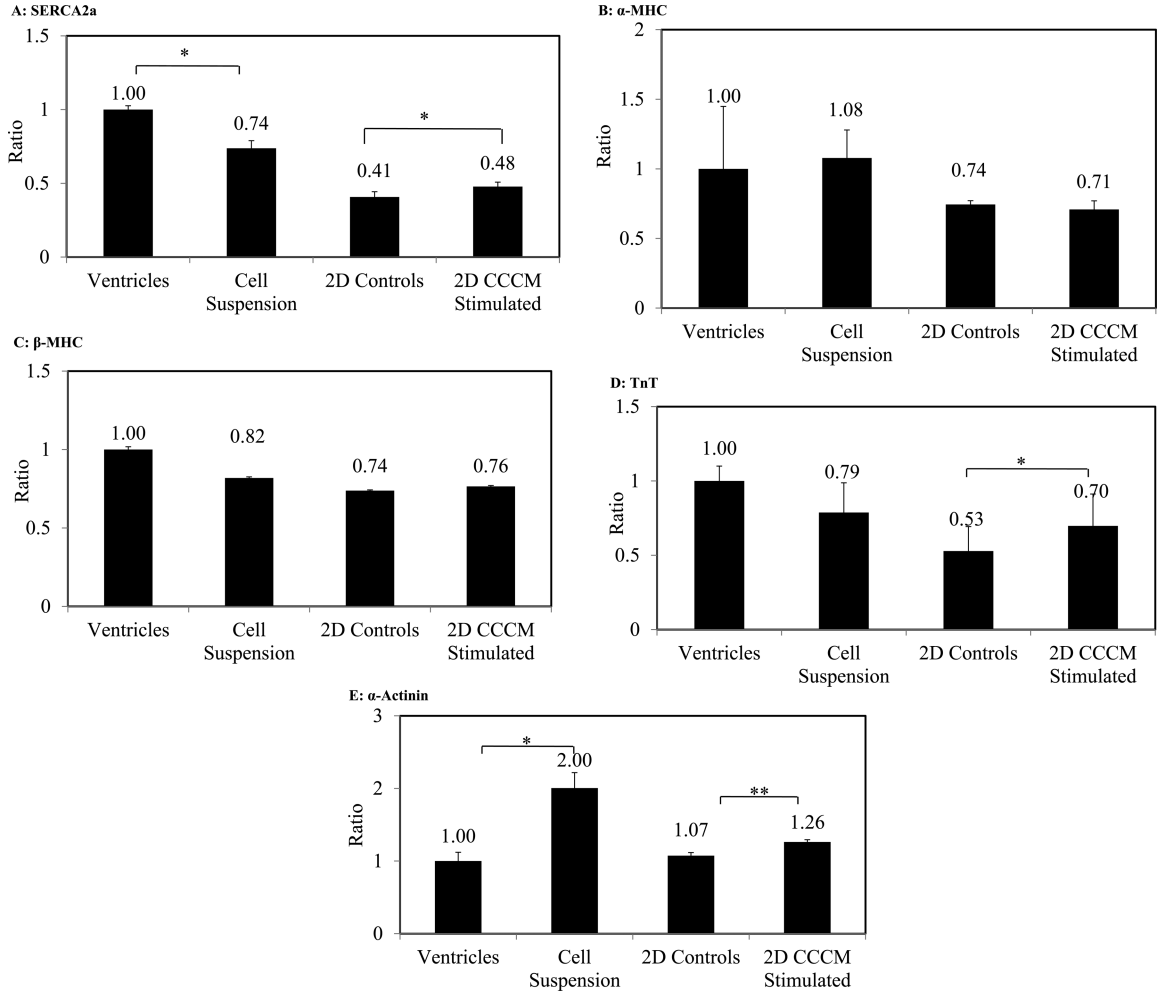

Figure 4. Graphs of interested protein with ventricular proteins set as the control state. (A) SERCA2a protein synthesis, (B) α-MHC protein synthesis, (C) β-MHC protein synthesis, (D) TnT protein synthesis, and (E) α-actinin protein synthesis. $N \geq 3$ and two tailed significance was set at $^{*}p < 0.05$ and $^{**}p < 0.005$.

Effect of Isoproterenol on Beat Rate (BR) and Contractility. The ISO effects on BR and contraction of the constructs cultured in the mechanically active CCCM system and in static conditions were observed. The BRs before and after each ISO application were recorded. The baseline BR before 0.5 μM ISO solution was used as the referent BR to determine the percentage change in BR in response to ISO (Figure 5). The results show that the BR of the CCCM group increased in response to increasing ISO doses to a maximal response at 10 μM ISO, then declined. In contrast, the BR of the control group showed no positive inotropic response to ISO and instead displayed a progressive decline in HR in response to increasing doses of ISO above 0.5 μM consistent with a toxic response.

Cardiac contractility with and without ISO was also examined. Movies of each ISO application were made, and maximum contraction was chosen to determine tissue shortening. Shortening was calculated as described in the previously publication.[5] With ISO applications, contractility of the stimulated tissues significantly increased versus the unstimulated group, to 7.55 $\pm$ 0.78% versus 4.52 $\pm$ 0.43%, respectively. In response to ISO, contractility increased 67% in the conditioned group versus 36% in the unconditioned group. These results suggest that mechanical stimulation may have accelerated CM maturation as measured by reduced toxicity and increased BR and shortening in response to ISO stimulation.

DISCUSSION

During cell isolation from the native 3D myocardium, cell−cell contacts are disrupted and extracellular matrix is destroyed, causing cells to lose extra cellular network connections that influence many cellular functions including calcium handling and contractility. Consistent with the negative impact of cell isolation is the disruption of gene and protein expressions of contractile relating proteins.[9] As outlined in the results, gene and protein expression of the Ca^{2+} regulating protein (SERCA2a) and contractile proteins (β-MHC) were lower in the cell suspension group and CCCM groups compared to the ventricle group. This may be due to the reduced metabolic activity of CM in 2D culture versus the in vivo state or the microenvironment of 3D engineered tissues. However, CM transcriptional and translational processes still occur and viable CM recover and mature in the CCCM. The proteins α-MHC obtained from the cell suspension showed higher than those in the ventricular tissues, but the p value shows no significant difference between these two groups. However, when these embryonic cardiac cells were cultured under 3D, gene expressions of α-MHC, β-MHC, and PLB increased versus

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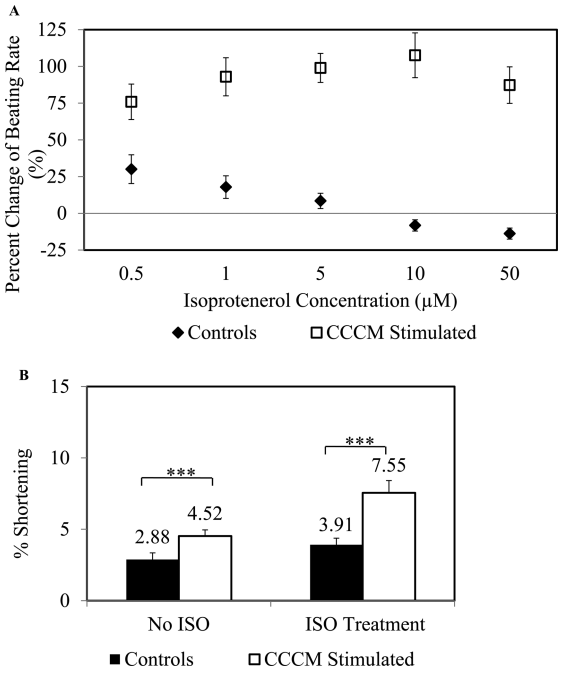

Figure 5. (A) Plot represents the percentage change in beat rate of the cardiac tissue under the effects of isoproterenol. While the control group showed cellular toxicity with increasing ISO concentration, the beat rate of the stimulated group increased and reached its peak at 10 μM ISO. (B) Changes in the cardiomyocyte contraction with and without the influence of ISO: controls (black) and CCCM stimulated (clear) constructs. Measurements were made at three different locations on each sample. $N = 6$. SEM was used for error bars; two tailed significance was set at ** $p < 0.05$ and ***$p < 0.001$.

CCCM culture, suggesting that 3D culture may be necessary for engineered cardiac tissues to maximally regulate transcriptional processes. Similar results have been noted using neonatal rat tissues.[14] While this study did not compare protein content or cellular toxicity in CCCM culture versus 3D engineered cardiac tissues, that comparison would provide insights into the regulation of CM maturation in 3D culture and is planned for future studies. It is also important to note that while in vitro measures of CM function (beat rate, force generation) and both gene and protein expression correlate with CM maturation, the ultimate measure of the ability of immature CM to augment cardiac repair requires in vivo implantation and functional analysis studies.

Different from all of the other CM associated proteins, α-actinin protein synthesis doubled in the short time from ventricle tissue isolation to cell suspension. In cardiac cells, α-actinin protein is the cytoskeletal actin-binding protein to stabilize the contractile apparatus.[10−12] Once the extra-cellular network was disrupted during cell isolation, increased α-actinin may be required to restore CM cell shape and integrity.

We noted SERCA2a protein synthesis increased in stimulated 2D CCCM constructs versus unstimulated controls; however, SERCA2a gene expression was similar. Embryonic chick CM are known to have immature sarcoplasmic reticulum (SR).[13] SERCA become fully functional only around birth and hence, limited adaptive differences in SERCA gene expression is to be expected in immature cardiomyocytes (days 10−15) before birth (day 21). Further, the level of PLB in the CCCM tissues was similar to static culture tissue, suggesting that cells did not undergo negative regulation in response to mechanical stimulation within the CCCM system. Finally, total RNA and protein obtained from the conditioned group was higher than controls, consistent with a positive effect of CCCM and conditioning on tissue maturation. Understanding these biomechanical responses of immature cardiac cells will also be important for engineering myocardial tissues from stem cell derived CM. It is also important to note that the increased maximal beat rate noted in the CCCM stimulated group may reflect increased CM ion channel maturation and studies that have used chronic pacing suggest improved functional CM maturation occurs at higher beat rates.

The two cardiac MHC isoforms reciprocally shift their expression depending on developmental stage and physiological conditions. β-MHC is the main ventricular MHC isoform prior to birth and then β-MHC decreases dramatically concurrent with increased α-MHC expression. No significant change in α- and β-MHC gene expression or protein synthesis occurred within the control and stimulated CCCM groups. Static β-MHC gene expression levels indicate that tissues undergoing mechanical treatment in the CCCM system were not overstressed, consistent with static PLB gene expression levels. Because this study was performed during embryonic and fetal stages, 5 days before hatching, α-MHC expression would not be expected to become dominant. Finally, the MHC isoform switch is regulated by thyroid hormone T3 levels which were not supplemented in the CCCM system.[14] Applying mechanical stimulation at an early stage of cardiac tissue development may not be enough to promote α- and β-MHC gene expression shifts or changes in protein profiles.[14,15] Therefore, thyroid hormone supplementation may be required to optimally mature CM within engineered tissues.

Catecholamine stimulation affects the mechanical performance of cardiac tissues through the activation of β-AR.[6,16,17] Under normal physiological conditions, catecholamine stimulation induces positive inotropic and lusitropic responses through a β1-AR-activated pathway. Once stimulation of β1-ARs via ISO binding occurs, cAMP accumulation rapidly increases which indirectly promotes the phosphorylation of PLB and enhances cardiac contractility. Subtype β1-ARs become dominant as CM mature under normal physiological conditions.[16,18] Under abnormal physiological condition of the heart, or heart failure, β1-ARs expression and function decreases but β2-ARs increases, leading to increased ISO related CM toxicity.[17]

After adding ISO, CCCM conditioned constructs yielded a higher percentage of contractions compared to the control ones; and the BR of the conditioned tissue increased until a 10 μM ISO solution (2.5 mg/mL) was added. However, for the controls, ISO concentrations higher than 10 μM created negative inotropy and reduced BR, ultimately resulting in noncontraction, similar to early studies from Serverin.[19] Thus, the increased tolerance of ISO and increased ISO mediated BR in the CCCM stimulated construct is consistent with mechanical conditioning mediated cell maturation. Higher BR and ISO resistance may be the result of higher cell−cell connection, more maturity, and/or higher cell density. Futher experiments are required to quantify the maturation of β1-adrenergic signaling in mechanically stimulated CCCM culture.

CONCLUSION

We have demonstrated that mechanical stimulation is needed to maintain CM proliferation and enhance protein synthesis of embryonic CM during embryogenesis. Mechanical stimulations

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also help to maintain the calcium regulation inside the cells. By providing a suitable mechanical stimulus for in vitro cardiac cultured tissues, our CCCM system holds significant promise for generation of functional cardiac patches for replacement of injured cardiac tissue.

FUTURE STUDIES

The CCCM system opens a wide opportunity to understanding the behavior of cardiac cells on molecular and cellular levels. It also provides a reliable tool to regenerate mature cardiac tissue for tissue transplants, which is our ultimate goal. Therefore, 3D constructs that incorporate mammalian CM lineages, such as human iPS-derived CM, and potentially incorporate both mechanical and electrical stimulation should be developed to optimize in vitro engineered tissues for cardiac repair and regeneration.

ASSOCIATED CONTENT

Supporting Information

Additional information on methods and discussion. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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