Hemodynamic Stimulation Using the Biomimetic Cardiac Tissue Model (BCTM) Enhances Maturation of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes

Aaron J. Rogers^a Ramaswamy Kannappan^b Hadil Abukhalifeh^d
Mohammed Ghazal^d Jessica M. Miller^b Ayman El-Baz^c Vladimir G. Fast^b
Palaniappan Sethu^a

^aDivision of Cardiovascular Disease, Departments of Medicine and Biomedical Engineering, University of Alabama at Birmingham, Birmingham, AL, USA; ^bDepartment of Biomedical Engineering, University of Alabama at Birmingham, Birmingham, AL, USA; ^cDepartment of Bioengineering, University of Louisville, Louisville, KY, USA; ^dCollege of Engineering, Abu Dhabi University, Abu Dhabi, UAE

Keywords

Cardiovascular tissue engineering · Hemodynamics · Hypertrophy · Stem cell differentiation

Abstract

Human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes (hiPSC-CMs) hold great promise for cardiovascular disease modeling and regenerative medicine. However, these cells are both structurally and functionally immature, primarily due to their differentiation into cardiomyocytes occurring under static culture which only reproduces biomolecular cues and ignores the dynamic hemodynamic cues that shape early and late heart development during cardiogenesis. To evaluate the effects of hemodynamic stimuli on hiPSC-CM maturation, we used the biomimetic cardiac tissue model to reproduce the hemodynamics and pressure/volume changes associated with heart development. Following 7 days of gradually increasing stimulation, we show that hemodynamic loading results in (a) enhanced alignment of the cells and extracellular matrix, (b)

significant increases in genes associated with physiological hypertrophy, (c) noticeable changes in sarcomeric organization and potential changes to cellular metabolism, and (d) a significant increase in fractional shortening, suggestive of a positive force frequency response. These findings suggest that culture of hiPSC-CMs under conditions that accurately reproduce hemodynamic cues results in structural organization and molecular signaling consistent with organ growth and functional maturation.

© 2019 S. Karger AG, Basel

Introduction

Cardiovascular disease is the number one cause of death in the USA and throughout the world, accounting for about 31% of all mortalities [Mozaffarian et al., 2016; Benjamin et al. 2018]. The high mortality rate associated with cardiovascular disease can be attributed to the limited intrinsic regenerative capacity of cardiomyocytes that make up the heart. Damage and death of cardiomyo-

cytes following events like myocardial infarctions result in the initiation of an inflammatory cascade of events that leads to cardiac tissue remodeling via cardiac fibroblast differentiation into myofibroblasts, and the infarcted areas being replaced with nonfunctional fibrotic scar tissue [Turner and Porter, 2013; Ma et al., 2014]. The resulting scar tissue does not contribute to contractile function and results in long-term complications that can result in sudden cardiac death, dilated cardiomyopathy, arrhythmias, and heart failure [Pauschinger et al., 1999; Khan and Sheppard, 2006; Dobaczewski et al., 2011; Louzao-Martinez et al., 2016].

In vitro studies play an important role in modeling diseases and developing drugs and therapeutics. The lack of appropriate human cell types to construct in vitro models of cardiac tissue has limited the relevancy of years of research into mechanisms and potential therapies. A vast majority of these studies used neonatal or embryonic cardiomyocytes from animal and avian sources [Martinsen, 2005; Warkman and Krieg, 2007; Miura and Yelon, 2011]. The use of adult cardiomyocytes is limited to acute studies due to the inability to maintain them in an in vitro culture without dedifferentiation [Bénardeau et al., 1997; Zhang et al., 2010]. The discovery of human induced pluripotent stem cells (hiPSCs) and the advent of protocols to efficiently differentiate them into cardiomyocytes (hiPSC-CMs) represents a major breakthrough in cardiovascular research that, for the first time, provided a human source of cardiomyocytes without the ethical concerns that accompany the use of embryonic stem cells. However, the relevance of hiPSC-CMs is limited, as they represent an immature state that more closely resembles fetal or embryonic cardiomyocytes that are structurally and functionally different from human adult cardiomyocytes [Bellin et al., 2012; Iglesias-García et al., 2013; Nakamura et al., 2013; Martins et al., 2014; Matsa et al., 2014; Feric and Radisic, 2016].

Cardiogenesis and cardiomyocyte maturation involve a highly orchestrated series of events that take place over an extended period of time and continue even after birth. The heart begins to beat and pump blood early in embryogenesis when it is still a linear tube with no formed valves or chambers [Clark and Hu, 1990; Keller et al., 1990]. This corresponds with cardiac differentiation of mesodermal precursors into immature cardiomyocytes. These immature cardiomyocytes first develop spontaneous action potentials, and then gradually begin contractions in a random and disorganized fashion sufficient to induce peristaltic blood flow within the linear heart tube. Gradually, these contractions become stronger and more

organized, resulting in small changes in pressure and stretch as blood flows through the linear heart tube. The various hemodynamic parameters like ventricular blood pressure, stroke volume, and heart rate increase in proportion to the weight of the embryo, such that blood flow indexed for the weight of the embryo remains constant [Clark et al., 1989; Hu and Clark, 1989]. During cardiogenesis, the heart is extremely sensitive to biomechanical cues, such that it will not develop properly in the absence of blood flow, and any alterations to normal hemodynamic stresses result in cardiac malformations and congenital heart defects [Rychter, 1978; Clark et al., 1984; Sedmera et al., 1999; Tobita and Keller, 2000]. Therefore, during cardiogenesis, blood flow and associated hemodynamic stresses play a critical role in the formation of the heart and the transformation of naïve cardiomyocytes into fully functional adult cardiomyocytes [Srivastava, 2006].

The dynamic environment and developmental cues associated with blood flow are contrary to the static environment in which hiPSC-CMs are typically differentiated which is devoid of any hemodynamic cues. Moreover, a majority of established protocols achieve differentiation in 2-dimensional (2D) monolayers. The 2D differentiation process under static conditions results in cells that have similar resting membrane potentials, sarcomeric proteins, ion channels, and mechanisms for calcium handling and storage to those in adult cardiomyocytes, but the cells are also smaller in size, exhibit underdeveloped and relatively disorganized sarcomeres, lack t-tubules, possess large pacemaker currents, and beat spontaneously [Itzhaki et al., 2006; O'Hara et al., 2011; Poon et al., 2011; Mummery et al., 2012; Synnergren et al., 2012; Robertson et al., 2013]. Using a 2D, biochemically driven process fails to replicate the complexity of the environment in vivo that relies on a combination of extracellular matrix (ECM) cues, electrical cues, and mechanical cues, and also the presence of and interaction with other noncardiomyocyte cell types (including endothelial cells and cardiac fibroblasts) required for cardiomyocyte maturation [Hirota et al., 1983; Hu and Clark, 1989; Clark and Hu, 1990; Hove et al., 2003; Moorman et al., 2003; Christoffels and Moorman, 2009; Bowers and Baudino, 2010]. Therefore, it is conceivable that culturing hiPSC-CMs while reproducing the hemodynamics of cardiogenesis may be the most efficient method of generating mature cardiomyocytes from these PSCs. Several groups have focused on mimicking geometric constraints [Pilarczyk et al., 2016], patterning [Carson et al., 2016], culture in dense collagen matrices [Roberts et al., 2016], culture in

3D fibrin gels [Zhang et al., 2013], and ontomimetic differentiation [Kerscher et al., 2016] to enable the progressive maturation of hiPSCs into hiPSC-CMs. While these efforts resulted in improvements in both the structural and functional maturation of hiPSC-CMs, levels of functional maturation associated with adult cardiomyocytes was not achieved. A recent work by Ronaldson-Bouchard et al. [2018] used progressively increased electromechanical conditioning to mature early-stage hiPSC-CMs and showed superior structural, functional, and metabolic maturation in comparison to other available methods. While this is an impressive achievement and moves us closer towards an adult cardiomyocyte, several functional measures including conduction velocities and contractile force generation fall short of what is seen in the adult myocardium. This could possibly be due to the fact that this process does not recreate the hemodynamic loading associated with gradual increases in pressure/volume loading seen as the heart transitions from a linear heart tube to a neonatal and then a postnatal heart.

To address the absence of hemodynamics in current protocols, we used the biomimetic cardiac tissue model (BCTM) which is the only system available that can recreate hemodynamic loading and unloading associated with pressure/volume changes in any chamber of the heart at any stage of development. We specifically focused on reproducing the blood flow associated with the early-stage heart. The novelty of this work is the ability to superimpose the flow, pressure, and stretch associated with critical events during early-stage heart development on iPSC-CMs during in vitro differentiation in a manner where the application of these stresses closely mimics what occurs in vivo. To further enhance physiological significance, we cocultured early-stage hiPSC-CMs (immediately following mesodermal induction and cardiac differentiation) and neonatal fibroblasts in a 3D fibrin gel to more closely mimic cardiac tissue.

Materials and Methods

Cell Culture Chamber Fabrication

Cell culture chambers were fabricated using standard soft-lithography using (poly)dimethyl siloxane (PDMS) (Sylgard 184, Dow Corning, Midland, MI, USA). The chamber was created using a two-step process of bonding a thin PDMS membrane to the bottom of a square PDMS block with an ellipse-shaped hole punched through the center measuring 15×30 mm. The PDMS block and thin membrane used a ratio of base-to-crosslinker of 10:1. The thin membrane was formed by spinning 10:1 PDMS to approximately 80 μ m on a silicon wafer within a tabletop centrifuge retro-fitted to hold and center the silicon wafers. Both components were cured

overnight at 60 °C. Once cured, both pieces were cleaned and bonded using oxygen plasma (Harrick Plasma Systems, Ithaca, NY, USA), optimized to create a permanent bond using 700 mTorr pressure, 30 W, and 20 s of exposure. Next, two 10:1 PDMS posts measuring 6.5 mm in height and 2 mm in diameter were placed on top of the thin membrane and along the ellipse's long axis 12 mm apart. The posts were bonded by placing the bonded PDMS chambers on top of a hot-plate set to 150 °C, and they were then dipped in liquid 10:1 PDMS and placed through a removable guide that allowed for consistent placement. After autoclaving, the chambers were filled with 2% hot liquid agarose and allowed to cool and solidify. Circles around each post and a center lane connecting these circles were punched out, leaving a dumb-bell shape within the chamber (Figure 1a). Finally, the chamber was ready for seeding with fibrin-encapsulated cells. The final product, once the gel was added and the agarose removed can be seen in Figure 1b.

Human iPSC-CM Culture and Differentiation

Human iPSCs were cultured within 6-well plates coated for 1 h at room temperature with hESC-qualified Matrigel supplied by Corning (354277) at the manufacturer-recommended concentration for maintaining pluripotency. The cells were cultured using MTeSR1 (Stemcell Technologies 85850) and receive daily media changes. At 75–85% confluency, the cells were disassociated using Accutase (Stemcell Technologies 07920), and then reseeded in MTeSR1 supplemented with 10 μ M Y-27632, ROCK1 inhibitor (Selleckchem S1049) at a split ratio of 1:6. After 12 h, hiPSCs were switched to MTeSR1 without ROCK1 inhibitor. After 3 days, hiPSCs reached a confluency of 75–85% and were switched to RPMI medium (Gibco 11875093) supplemented with B27 without insulin (Gibco A18956-01) and 10 μ M WNT promoter CHIR99021 (Stemcell Technologies 72052), considered day 1 of differentiation. After exactly 24 h, the cells were washed and switched to media without CHIR99021. They were maintained with daily media changes until day 4, when the media was supplemented with 5 μ M WNT inhibitor IWP-2 (Stemcell Technologies 72124). They were cultured with IWP-2 until day 6. On day 8, the cells were switched to RPMI medium supplemented with B27 with insulin (Gibco 17504044). At this point, the cells were referred to as hiPSC-CMs and had begun spontaneously beating. Medium was changed daily until day 10, and refreshed thereafter every 2 days. Only plates that contained >60% beating hiPSC-CMs were used for further study.

Human Primary Fibroblast Culture

Neonatal primary dermal fibroblasts were purchased from ATCC (PCS-201-010) and cultured in primary fibroblast medium (ATCC PCS-201-030) supplemented with Fibroblast Growth Kit-Low serum (ATCC PCS-201-041). Cells were cultured according to ATCC recommendations and were not used in any study past passage 15.

Fibrin Gel Encapsulation

On day 12, the hiPSC-CMs were disassociated by using 0.25% Trypsin/EDTA and combined with human fibroblasts disassociated by using 0.05% Trypsin/EDTA at a final density of 2.0×10^6 fibroblasts and 4.0×10^6 hiPSC-CMs per 300 μ L of fibrin solution. The fibrin solution was composed of DMEM-HG supplemented with 10% FBS, 50 μ g/mL L-ascorbic acid (Sigma A5960), 1 $\times$ non-essential amino acids (Gibco 11140-050), and 5 mg/mL aminoca-

(For legend see next page.)

proic acid (Acros 103301000), and contained a final concentration of 2 mg/mL fibrinogen (Millipore 341576), 0.9 units thrombin (Millipore 605195)/mg of fibrin (0.54 units/gel), and 2 mM CaCl₂. The fibrin-encapsulated cells were then placed within a 5% CO₂ incubator for 30 min at 37 °C to complete the fibrin polymerization. Following polymerization and until the end of experimentation, the cells were maintained with DMEM-HG supplemented with 10% FBS, 50 µg/mL ascorbic acid, 1× nonessential amino acids, and 5 mg/mL aminocaproic acid. Once within the fibrin gel, the cells were maintained by daily media changes. Cells were allowed to spread and adapt to the 3D architecture as well as recover from the trypsinization and encapsulation process for 5 days before beginning BCTM stimulation.

BCTM Stimulation

The mechanism of action of the BCTM revolves around using dynamic pressure differences above and below the cell culture chamber membrane to reproduce the cardiac cycle [Rogers et al., 2016]. The BCTM consists of media reservoir set to a specified height to create a downward filling pressure imposed onto the cell culture member to mimic a diastolic filling pressure. A programmable pneumatic pump is used to increase the pressure below the cell culture membrane to generate a systolic pressure. The cycling of the pneumatic pump coupled with one-way flow control valves allows the actuation of the culture membrane to displace fluid in one direction with each contraction (Fig. 1e). The BCTM has changed from its previous published iterations through its adaptation to the culture of 3D tissue fibers suspended between 2 posts (Fig. 1b). Another notable difference in the current design is the presence of a dome-shaped insert below the cell culture chamber and aligned between the 2 posts, that deforms the membrane as it is pushed down onto the insert during the diastole phase of the cycle. By recording the post movements during the cardiac cycle (Fig. 1c, d), it is possible to calculate strain.

BCTM Stimulation Conditions

To adapt to the mechanical stress, the cells are placed under gentle conditions and ramped up over time (Fig. 2). Gradually increasing the stress was found to be necessary for hiPSC-CMs to survive under hemodynamic loading [Rogers et al., 2016]. On day

0, the stimulation was set at a peak systolic pressure of 5 mm Hg, an end-diastolic pressure of 1 mm Hg, and a maximum strain of 0.2%. These were increased to 10 mm Hg, 3 mm Hg, and 0.7%, respectively, after 24 h (day 1); 15 mm Hg, 5 mm Hg, and 1.2%, respectively, on day 2; and 30 mm Hg, 10 mm Hg, and 2.3%, respectively, on day 3. This final condition was maintained until the end of the 7-day experiment. Throughout the entire experiment, the cycle frequency was maintained at 1 Hz so as to replicate a normal human resting heart rate. The pressure and stretch stimulus were determined to ensure cell survival; higher levels of mechanical stimulation have been found to be detrimental to cell survival. The 7-day stimulation represented the initial phase of embryonic heart development during which blood flow and pressure/volume changes are initiated. Also, 7 days is sufficient time to clearly see changes in cardiomyocyte structural and functional maturation.

Staining

At the end of the experiment, the fibrin-encapsulated cells were removed from the BCTM, and then fixed using fresh 4% paraformaldehyde at room temperature for 1 h before moving to 4 °C. Samples were then placed in OCT solution and frozen for cryosectioning of 10-µm-thick slices. Sections were immunolabeled with rabbit anti-cardiac troponin T (anti-cTnT; Abcam, ab91605) and mouse monoclonal anti-α sarcomeric actin (anti-α-SA) antibody (Sigma, A2172). Nuclei were counterstained with DAPI. Images were taken using an Olympus IX83 epifluorescence microscope.

Transmission Electron Microscopy

At the end of the 7-day stimulation protocol, the cell fibers were removed from the BCTM, and then fixed using a fixative composed of 2% paraformaldehyde and 2.5% glutaraldehyde in a 0.1-M sodium cacodylate solution for 1 h at room temperature, and then moved to 4 °C overnight. Samples were then taken to the UAB high-resolution imaging facility where they were further processed, sliced, embedded, and sectioned. Slices were imaged using a Tecnai Spirit T12 transmission electron microscope.

RNA qRT-PCR

After 7 days of stimulation, the cells were removed from the BCTM, carefully slid off the posts, placed within a liquid nitrogen safe cryovial with 700 µL Trizol (Invitrogen), and then flash-frozen and maintained in liquid nitrogen until processing. Once enough samples had been collected for gene analysis, they were thawed at room temperature and immediately placed within a beadmill (BeadBug D1030) containing 1.0 mm silica beads (Benchmark D1031-10) and milled for 30 s at speed 4,000. RNA was then isolated using standard phenol-chloroform extraction methods for quantification of the transcripts GADPH, GATA4, NKX2.5, MYLK, MYL2, KCNH2, CAMK2B, and MYH7. Complementary DNA for mRNAs was obtained from 2 µg total RNA in a 20-µL reaction using TaqMan reverse-transcription reagents (Thermo Fisher Scientific) and 100 pmol of oligo(dT)15 primer. This mixture was incubated at 37 °C for 2 h. Quantitative RT-PCR was performed with primers (Table 1) using the Eppendorf RealPlex² real-time PCR system. Complementary DNA synthesized from 100 ng total RNA was combined with Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific) and 0.5 µM each of the forward and reverse primers. Cycling conditions were as follows: 95 °C for 10

Fig. 1. a BCTM cell culture chamber with agarose mold prior to the addition of cells within the fibrin gel. **b** BCTM cell culture chamber with the cell-laden fibrin gel suspended between the 2 posts. This image was taken 2 days after removing the agarose mold. The gel originally takes the shape of the agarose mold but contracts over time as shown in the image. **c** The position of the posts within the cell culture chamber at the end of systole. The fiber experiences no strain at this point as the posts are at the initial resting position. **d** Position of posts within the cell culture chamber at the end of diastole. The thin membrane contacts with the insert below and deforms along its surface, thus pulling the posts away from each other and applying uniaxial strain to the fiber. **e** The diagram represents how the BCTM reproduces the cardiac cycle. The BCTM imposes uniaxial strain onto the cells by using a flexible membrane that is stretched over an arched surface placed below the membrane. Yellow indicates the cell-laden fibrin fiber.

Table 1. Genes with corresponding forward and reverse primers used for the qRT-PCR

Gene	Forward primer	Reverse primer
MYH7	GAGGACAAGGTCAACACCT	CGCACCTTCTTCTCTTGCTC
MYLK	AGTTGTGGAGGGAAGTGCTG	GGACTCCCTGATTGACTGGT
CAMK2B	CACCGACGAGTACCAGCTCT	GTTGATGATCTTGGCTGCAT
GAPDH	AAGGTGAAGGTCGGAGTCAA	AATGAAGGGGTCATTGATGG
GATA4	TCTAAGACACCAGCAGCTCCTTCA	ACTGACTGAGAACGCTCGGGACAC
NKX2.5	CACCATGGGGAGAAAAAAGATTCA	TGTTGCCCATCATTAGAAAAGTC
MYL2	CCAACTCCAACGTGTTCTCCATGT	CATCAATTTCTTCATTTTTCACGTTCA
KCNH2	GGGAGCTTGGGGCCTGACC	TCCCCTCCCCCGCCTCACAC

Fig. 2. Pressure/volume loops that hiPSC-CMs were subjected to during the 7-day study. The systolic/diastolic ratio was maintained at 40/60% and the frequency at 1 Hz throughout the duration of the experiment.

min followed by 40 cycles of amplification (95°C denaturation for 15 s, and 60°C annealing-extension for 1 min). To avoid the influence of genomic contamination, the forward and reverse primers for each gene were located in different exons. Relative quantitative expression of genes of interest was calculated after normalizing to the housekeeping gene GAPDH.

Fractional Shortening

Immediately after removing the hiPSC-CM fibers from the BCTM, their intrinsic (spontaneous) and paced contractions at 1.0, 1.3, 1.5, and 2.0 Hz were recorded using a video camera. A Myopacer field stimulator (Ionoptix) with adapted carbon electrodes provided field stimulation at 4.0 V/cm with a 40-ms duration. The field was set to alternate polarity to avoid build-up on the electrodes. The carbon electrodes were placed near the bottom of the cell culture surface and between the post and outside edge of the cell culture chamber so that the field ran along the entire length

of the fiber. HBSS (Gibco 14025–092) warmed to 37°C was used during the recordings.

Fractional shortening was estimated using video analysis and Lagrangian strain calculations for finitely small displacement, as previously described by Bonet and Wood [2008] and used by our group [Nguyen et al., 2009, 2015]. Briefly, if p_1 and p_2 are 2 nearby points defined on the first frame and d_1 denotes the Euclidian distance between p_1 and p_2 at the first frame (i.e., reference frame), then d_j denotes the Euclidian distance between these 2 points at time frame j . The engineering strain S , at frame j , is then defined as: $S_j = (d_j - d_1)/d_1$.

A custom algorithm was written to estimate planar Lagrangian strains. First, the peak displacement was determined by tracking 2 points based on their appearance features through the image frames, and then calculating the displacement between the 2 points over the image frames. The maximum displacement was then selected as the reference frame. Given that the contractions are unidirectional due to alignment of the fibers, 2 pairs of points on the first (reference) frame were determined and their tracked pairs on the selected frame with maximum displacement. Lagrangian strain for each of the points was estimated using the previously mentioned equation. Finally, the mean Lagrangian strain was calculated and expressed as % shortening. To account for drift due to macroscopic movement of the fiber, the analysis was performed on a per-beat basis where the measurements correspond to maximum and minimum displacements per contraction.

Statistical Analysis

An unpaired t test was used for the analysis of the gene expression and fractional shortening data with two-tailed significance set at $p \leq 0.05$ for both experiments.

Results

Cardiomyocyte Sarcomeric Structure

Immunofluorescence microscopy was performed to visualize sarcomeric organization in both stimulated and static samples. Images show that both stimulated and static samples had organized sarcomeres with striated organization of the α -SA and cTnT (Fig. 3).

Fig. 3. **a** Static controls. **b** BCTM-stimulated samples with the entire fiber stained with cardiac troponin T (cTnT), α sarcomeric actin (α -SA), and DAPI with magnified images of α -SA (bottom), cTnT (middle), and in combination (top).

Cardiomyocyte Ultrastructural Morphology

Transmission electron microscopy (TEM) was performed to evaluate changes in intracellular and extracellular structure and organization. Images clearly show that the ECM associated with the stimulated samples is more aligned and composed of longer, thicker fibrils (Fig. 4b, top) in comparison to the shorter, thinner, and more disorganized fibrils in static controls (Fig. 4a, top). Static control cells were inundated with lipid deposits and unorganized regions of sarcomeric proteins (denoted with a black and white arrow, respectively; Fig. 4, middle, left) whereas the stimulated samples did not show lipid depos-

its but had more highly organized sarcomeres (Fig. 4b, middle). A highly magnified image of sarcomeres shows that Z-disks, A-bands, and I-bands are clearly visible in both the static controls and stimulated samples (Fig. 4, bottom) with evidence of the beginnings of the formation of an M-line in the stimulated sample (black arrow).

Expression of Genes Involved in Structural and Functional Maturation

Static and stimulated samples were profiled for changes in genes associated with cardiomyocyte hypertrophy and cardiac-specific structural maturation (Fig. 5). Sev-

Fig. 4. a Static controls. **b** Stimulated samples. Top: visualization of ECM. Middle: images show sarcomeres and associated structures within cardiomyocytes. **a** The black arrow indicates lipid deposits and the white arrow indicates an example of unorganized sarcomeres, both of which were common throughout the static controls. Bottom: high-magnification images of representative sarcomeres. **b** The black arrow marks a white area along the middle of the sarcomere that was found consistently in the stimulated sarcomeres and what could be the formation of the M-line.

Fig. 5. Static controls versus BCTM-stimulated (Stim) samples normalized to GAPDH expression levels. Statistically significant upregulation was observed in the genes *GATA4*, *MYL2*, *CAM2KB*, and *MYH7* in stimulated ($n = 5$) versus static ($n = 4$) samples. * $p \leq 0.05$

eral genes associated with hypertrophic growth were up-regulated in the stimulated samples. Our results confirm that *GATA4*, myosin regulatory light chain 2 (*MYL2*), calcium/calmodulin-dependent protein kinase type II β chain (*CAMK2B*), and myosin heavy chain β (*MYH7*) were significantly upregulated in stimulated samples versus in the static controls. We also found no statistically significant changes in *NKX2.5*, myosin light chain kinase (*MYLK*), and potassium voltage-gated channel subfamily

H member 2 (*KCNH2*). Although a mixed population of fibroblasts and cardiomyocytes make up the cell samples used for gene analysis, the genes chosen were highly cardiac specific.

Functional Maturation of Cardiomyocytes

Video analysis of the fractional shortening shows that stimulated samples achieved greater fractional shortening than the samples cultured under static conditions. It

Fig. 6. Data collected from videos made of the fibers undergoing electrical stimulation at the frequencies 1, 1.3, 1.5, and 2.0 Hz. Videos were also made of the intrinsic (spontaneous) fractional shortening of each fiber ($n = 4$). The stimulated samples (Stim) showed significantly higher fractional shortening at 1.3, 1.5, and 2.0 Hz. The intrinsic contractions were not significantly different. * $p \leq 0.05$.

can also be noted that the static samples demonstrated a decrease in fractional shortening at progressively higher pacing frequencies, while that of the stimulated samples peaked at the 1.3-Hz pacing frequency before progressively decreasing at higher pacing frequencies (Fig. 6). Importantly, significantly higher fractional shortening was measured in the stimulated samples ($n = 6$) in comparison to the static samples ($n = 5$) at the frequencies 1.3, 1.5, and 2.0 Hz.

Discussion/Conclusion

Given the importance of mechanical loading during embryonic heart development, we sought to determine if hemodynamic stimulation could potentially impact the differentiation of hiPSC-CMs into a more mature phenotype. This involved the culture of early-stage (immediately following cardiac differentiation of mesodermal precursors) hiPSC-CMs under peristaltic flow and pressure, stretch which is gradually increased to 20 mm Hg, and 3% stretch within the BCTM. The results provide preliminary validation that recreating mechanical loading during early cardiac differentiation induces

changes associated with the stage of embryonic heart development linked to an increase in chamber size and functional maturation. Immunofluorescence microscopy provided clear evidence of physiological hypertrophy and enhanced organization of hiPSC-CMs subject to stimulation within the BCTM (Fig. 3). Visualization of cTnT and α -SA showed significant improvements in the sarcomeric alignment and localization of actin and troponin in clear, alternating patterns in the stimulated samples, but not in the static controls. It also appears that each sarcomere was wider and longer than the sarcomeres in the static controls, although this was not quantified.

Examination of ultrastructural morphology using TEM revealed important distinctions between the static controls and stimulated samples (Fig. 4). It appears that mechanical stimulation greatly enhanced the alignment of the ECM and enabled the formation of longer and thicker ECM fibrils. This is potentially beneficial to cardiomyocyte maturation by providing alignment cues and coordinating force transmission along the length of the engineered muscle fiber. Stimulation also increased the overall alignment of sarcomeres and prevented disorganized regions that were observed in the static controls. High-magnification images showed organization of sarcomeres including Z-disks, A-bands, and I-bands in both static controls and stimulated samples. Closer examination showed the beginnings of the formation of M-lines in the stimulated samples. Surprisingly, we found lipid deposits within hiPSC-CMs in the static controls. The absence of lipid deposits in the stimulated samples could possibly be explained by a switch to fatty acid metabolism due to the higher energetic demand of stimulated cells to achieve physiological hypertrophy and growth. However, at this stage of development (embryonic/neonatal), glycolysis is still the primary source of energy associated with proliferation and organ growth. Additional studies are necessary to determine the significance of lipid accumulation in the static controls and evaluate possible differences in metabolic activity between the static controls and stimulated samples. TEM is commonly used to evaluate ultrastructure morphology of cardiomyocytes. Adult cardiomyocytes are defined by organized bands and lines (A-band, M-line, Z-line, and I-bands) that make up the sarcomere and are flanked by sarcoplasmic reticulum and a high number of mitochondria that typically run along the length of the sarcomere. The presence of each band along with increased alignment places our stimulated samples closer to what would be expected from fetal cardiac tissue [Ronaldson-

Bouchard et al., 2018], which confirms that there is early and progressive maturation, supporting our hypothesis.

Gene expression studies provide further evidence of physiological hypertrophy in the BCTM-stimulated samples (Fig. 5). The upregulation of *GATA4* is significant due to its presence in developing and adult cardiomyocytes where it has been linked to hypertrophic growth of both immature and mature cardiomyocytes. *MYL2* and *MYH7* are both part of the myosin complex involved in binding to actin filaments and are integral to proper contractile function. Upregulation of these 2 genes implies the creation of new sarcomeric structures and possibly explains the increase in fractional shortening observed in the stimulated samples. Human *CAMK2B* is a calcium-handling protein that was upregulated in the stimulated samples, and this suggests improved calcium handling and hypertrophy. The lack of a change in *KCNH2* could be due to the stimulation period being too short or that electrical stimulation may also be required for upregulation of voltage-regulated channels, as seen in other studies using electrical stimulation for maturation [Eng et al., 2016].

Finally, the fractional shortening measurements suggested significant improvements in percent shortening of stimulated samples in comparison to static controls (Fig. 6). Interestingly, the fractional shortening data suggests that stimulation within the BCTM induced a positive force frequency response in the stimulated samples, i.e., an increase in contractile force with increasing frequency. The stimulated samples demonstrated a peak fractional shortening at 1.3 Hz which gradually decreased with increasing frequencies, creating a bell curve indicative of the force frequency response measured in mature cardiac tissue. A similar bell curve peaking at 1.5 Hz is present in normal healthy cardiac tissue. It is possible that this is an example of a positive force frequency response, but it should be noted that the increases in fractional shortening between pacing frequencies in the stimulated samples were not significantly different. However, it is clear that the fractional shortening was significantly increased in the stimulated samples when compared to the static culture at most pacing frequencies.

In summary, the BCTM was used to successfully recreate hemodynamic loading associated with early heart development. 3D-engineered cardiac tissue fibers constructed using early-stage hiPSC-CMs/neonatal fibroblast cocultures stimulated for 7 days within the BCTM showed noticeable and quantifiable changes in structure,

alignment, organization, hypertrophic gene expression, and function when compared to engineered fibers maintained under static culture. These results suggest that the BCTM can be used to recreate hemodynamic cues associated with embryonic development and could possibly be used in conjunction with electrical stimulation to mimic conditions associated with the postnatal (adult) heart.

Acknowledgements

We would like to acknowledge the National Institute of Health, University of Alabama at Birmingham Comprehensive Cardiovascular Center, and University of Alabama at Birmingham Department of Biomedical Engineering for giving us the funds to conduct this research. We would also like to thank the UAB TEM core facility for their expertise in getting the TEM figure.

Statement of Ethics

All authors followed standard ethical procedures. No studies involved the use of animal or human subjects.

Disclosure Statement

The authors have no conflicts of interest to declare.

Funding Sources

This work was funded by a National Institute of Health R21 grant # 11675980, funds from the University of Alabama at Birmingham Comprehensive Cardiovascular Center and Department of Biomedical Engineering. A.J.R. was supported by NIH T32 training grant # 5T32HL007918-18.

Author Contributions

P.S. was involved with the overall study design. A.J.R. performed all iPSC culture and differentiation, BCTM stimulation, was involved in all steps of analysis, and wrote most of this article. R.K. and J.M.M. performed sectioning, immunolabeling and confocal imaging, and RT-PCR based RNA analysis. H.A., M.G., and A.E. enabled fractional shortening analysis. V.F. was consulted to develop the electrical stimulation protocol and apparatus. All authors contributed towards the writing and editing of the manuscript.

References

- Bellin M, Marchetto MC, Gage FH, Mummery CL. Induced pluripotent stem cells: the new patient? *Nat Rev Mol Cell Biol*. 2012 Nov; 13(11):713–26.
- Bénardeau A, Hatem SN, Rücker-Martin C, Tessier S, Dinanian S, Samuel JL, et al. Primary culture of human atrial myocytes is associated with the appearance of structural and functional characteristics of immature myocardium. *J Mol Cell Cardiol*. 1997 May;29(5):1307–20.
- Benjamin EJ, Virani SS, Callaway CW, Chamberlain AM, Chang AR, Cheng S, et al; American Heart Association Council on Epidemiology and Prevention Statistics Committee and Stroke Statistics Subcommittee. Heart Disease and Stroke Statistics. 2018 Update: A Report from the American Heart Association. *Circulation*. 2018 Mar;137(12):e67–492.
- Bonet J, Wood RD, editors. *Nonlinear Continuum Mechanics for Finite Element Analysis*. Cambridge: University Press; 2008.
- Bowers SLK, Baudino TA. Laying the groundwork for growth: cell-cell and cell-ECM interactions in cardiovascular development. *Birth Defects Res C Embryo Today*. 2010 Mar; 90(1):1–7.
- Carson D, Hnilova M, Yang X, Nemeth CL, Tsui JH, Smith AS, et al. Nanotopography-Induced Structural Anisotropy and Sarcomere Development in Human Cardiomyocytes Derived from Induced Pluripotent Stem Cells. *ACS Appl Mater Interfaces*. 2016 Aug;8(34):21923–32.
- Christoffels VM, Moorman AF. Development of the cardiac conduction system: why are some regions of the heart more arrhythmogenic than others? *Circ Arrhythm Electrophysiol*. 2009 Apr;2(2):195–207.
- Clark EB, Hu N. Hemodynamics of the developing cardiovascular system. *Ann N Y Acad Sci*. 1990;588:41–7.
- Clark EB, Hu N, Frommelt P, Vandekieft GK, Dummett JL, Tomanek RJ. Effect of increased pressure on ventricular growth in stage 21 chick embryos. *Am J Physiol*. 1989 Jul;257(1 Pt 2):H55–61.
- Clark EB, Hu N, Rosenquist GC. Effect of conotruncal constriction on aortic-mitral valve continuity in the stage 18, 21 and 24 chick embryo. *Am J Cardiol*. 1984 Jan;53(2):324–7.
- Dobaczewski M, Chen W, Frangogiannis NG. Transforming growth factor (TGF)- β signaling in cardiac remodeling. *J Mol Cell Cardiol*. 2011 Oct;51(4):600–6.
- Eng G, Lee BW, Protas L, Gagliardi M, Brown K, Kass RS, et al. Autonomous beating rate adaptation in human stem cell-derived cardiomyocytes. *Nat Commun*. 2016 Jan;7(1):10312.
- Feric NT, Radisic M. Maturing human pluripotent stem cell-derived cardiomyocytes in human engineered cardiac tissues. *Adv Drug Deliv Rev*. 2016 Jan;96:110–34.
- Hirota A, Sakai T, Fujii S, Kamino K. Initial development of conduction pattern of spontaneous action potential in early embryonic precontractile chick heart. *Dev Biol*. 1983 Oct; 99(2):517–23.
- Hove JR, Köster RW, Forouhar AS, Acevedo-Bolton G, Fraser SE, Gharib M. Intracardiac fluid forces are an essential epigenetic factor for embryonic cardiogenesis. *Nature*. 2003 Jan;421(6919):172–7.
- Hu N, Clark EB. Hemodynamics of the stage 12 to stage 29 chick embryo. *Circ Res*. 1989 Dec; 65(6):1665–70.
- Iglesias-García O, Pelacho B, Prósper F. Induced pluripotent stem cells as a new strategy for cardiac regeneration and disease modeling. *J Mol Cell Cardiol*. 2013 Sep;62:43–50.
- Itzhaki I, Schiller J, Beyar R, Satin J, Gepstein L. Calcium handling in embryonic stem cell-derived cardiac myocytes: of mice and men. *Ann N Y Acad Sci*. 2006 Oct;1080(1):207–15.
- Keller BB, Hu N, Clark EB. Correlation of ventricular area, perimeter, and conotruncal diameter with ventricular mass and function in the chick embryo from stages 12 to 24. *Circ Res*. 1990 Jan;66(1):109–14.
- Kerscher P, Turnbull IC, Hodge AJ, Kim J, Seliktar D, Easley CJ, et al. Direct hydrogel encapsulation of pluripotent stem cells enables on-tomimetic differentiation and growth of engineered human heart tissues. *Biomaterials*. 2016 Mar;83:383–95.
- Khan R, Sheppard R. Fibrosis in heart disease: understanding the role of transforming growth factor-beta in cardiomyopathy, valvular disease and arrhythmia. *Immunology*. 2006 May;118(1):10–24.
- Louza-Martinez L, Vink A, Harakalova M, Asseberg FW, Verhaar MC, Cheng C. Characteristic adaptations of the extracellular matrix in dilated cardiomyopathy. *Int J Cardiol*. 2016 Oct;220:634–46.
- Ma Y, de Castro Brás LE, Toba H, Iyer RP, Hall ME, Winniford MD, et al. Myofibroblasts and the extracellular matrix network in post-myocardial infarction cardiac remodeling. *Pflugers Arch*. 2014 Jun;466(6):1113–27.
- Martins AM, Vunjak-Novakovic G, Reis RL. The current status of iPS cells in cardiac research and their potential for tissue engineering and regenerative medicine. *Stem Cell Rev*. 2014 Apr;10(2):177–90.
- Martinsen BJ. Reference guide to the stages of chick heart embryology. *Dev Dyn*. 2005 Aug; 233(4):1217–37.
- Matsa E, Burrage PW, Wu JC. Human stem cells for modeling heart disease and for drug discovery. *Sci Transl Med*. 2014;6(239):239.
- Miura GI, Yelon D. A guide to analysis of cardiac phenotypes in the zebrafish embryo. *Methods Cell Biol*. 2011;101:161–80.
- Moorman A, Webb S, Brown NA, Lamers W, Anderson RH. Development of the heart: (1) formation of the cardiac chambers and arterial trunks. *Heart*. 2003 Jul;89(7):806–14.
- Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, et al; Writing Group Members; American Heart Association Statistics Committee; Stroke Statistics Subcommittee. Heart Disease and Stroke Statistics. 2016 Update: A Report From the American Heart Association. *Circulation*. 2016 Jan; 133(4):e38–360.
- Mummery CL, Zhang J, Ng ES, Elliott DA, Elfant AG, Kamp TJ. Differentiation of human embryonic stem cells and induced pluripotent stem cells to cardiomyocytes: a methods overview. *Circ Res*. 2012 Jul;111(3):344–58.
- Nakamura K, Hirano K, Wu SM. iPS cell modeling of cardiometabolic diseases. *J Cardiovasc Transl Res*. 2013 Feb;6(1):46–53.
- Nguyen MD, Giridharan G, Prabhu SD, Sethu P. Microfluidic cardiac circulation model (microCCM) for functional cardiomyocyte studies. *Conf Proc IEEE Eng Med Biol Soc*. 2009; 2009:1060–3.
- Nguyen MD, Tinney JP, Ye F, Elnakib AA, Yuan F, El-Baz A, et al. Effects of physiologic mechanical stimulation on embryonic chick cardiomyocytes using a microfluidic cardiac cell culture model. *Anal Chem*. 2015 Feb;87(4):2107–13.
- O'Hara T, Virág L, Varró A, Rudy Y. Simulation of the undiseased human cardiac ventricular action potential: model formulation and experimental validation. *PLOS Comput Biol*. 2011 May;7(5):e1002061.
- Pauschinger M, Knopf D, Petschauer S, Doerner A, Poller W, Schwimmbeck PL, et al. Dilated cardiomyopathy is associated with significant changes in collagen type I/III ratio. *Circulation*. 1999 Jun;99(21):2750–6.
- Pilarczyk G, Raulf A, Gunkel M, Fleischmann BK, Lemor R, Hausmann M. Tissue-Mimicking Geometrical Constraints Stimulate Tissue-Like Constitution and Activity of Mouse Neonatal and Human-Induced Pluripotent Stem Cell-Derived Cardiac Myocytes. *J Funct Biomater*. 2016 Jan;7(1):1.
- Poon E, Kong CW, Li RA. Human pluripotent stem cell-based approaches for myocardial repair: from the electrophysiological perspective. *Mol Pharm*. 2011 Oct;8(5):1495–504.
- Roberts MA, Tran D, Coulombe KL, Razumova M, Regnier M, Murry CE, et al. Stromal Cells in Dense Collagen Promote Cardiomyocyte and Microvascular Patterning in Engineered Human Heart Tissue. *Tissue Eng Part A*. 2016 Apr;22(7–8):633–44.
- Robertson C, Tran DD, George SC. Concise review: maturation phases of human pluripotent stem cell-derived cardiomyocytes. *Stem Cells*. 2013 May;31(5):829–37.
- Rogers AJ, Fast VG, Sethu P. Biomimetic Cardiac Tissue Model Enables the Adaptation of Human Induced Pluripotent Stem Cell Cardiomyocytes to Physiological Hemodynamic Loads. *Anal Chem*. 2016 Oct;88(19):9862–8.

- Ronaldson-Bouchard K, Ma SP, Yeager K, Chen T, Song L, Sirabella D, et al. Advanced maturation of human cardiac tissue grown from pluripotent stem cells. *Nature*. 2018 Apr; 556(7700):239–43.
- Rychter Z. Analysis of relations between aortic arches and aorticopulmonary septation. *Birth Defects Orig Artic Ser*. 1978;14(7):443–8.
- Sedmera D, Pexieder T, Rychterova V, Hu N, Clark EB. Remodeling of chick embryonic ventricular myoarchitecture under experimentally changed loading conditions. *Anat Rec*. 1999 Feb;254(2):238–52.
- Srivastava D. Genetic regulation of cardiogenesis and congenital heart disease. *Annu Rev Pathol*. 2006;1(1):199–213.
- Synnergren J, Améen C, Jansson A, Sartipy P. Global transcriptional profiling reveals similarities and differences between human stem cell-derived cardiomyocyte clusters and heart tissue. *Physiol Genomics*. 2012 Feb;44(4): 245–58.
- Tobita K, Keller BB. Right and left ventricular wall deformation patterns in normal and left heart hypoplasia chick embryos. *Am J Physiol Heart Circ Physiol*. 2000 Sep;279(3):H959–69.
- Turner NA, Porter KE. Function and fate of myofibroblasts after myocardial infarction. *Fibrogenesis Tissue Repair*. 2013 Mar;6(1):5.
- Warkman AS, Krieg PA. Xenopus as a model system for vertebrate heart development. *Semin Cell Dev Biol*. 2007 Feb;18(1):46–53.
- Zhang D, Shadrin IY, Lam J, Xian HQ, Snodgrass HR, Bursac N. Tissue-engineered cardiac patch for advanced functional maturation of human ESC-derived cardiomyocytes. *Biomaterials*. 2013 Jul;34(23):5813–20.
- Zhang Y, Li TS, Lee ST, Wawrowsky KA, Cheng K, Galang G, et al. Dedifferentiation and proliferation of mammalian cardiomyocytes. *PLoS One*. 2010 Sep;5(9):e12559.