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Bioengineering an electro-mechanically functional miniature ventricular heart chamber from human pluripotent stem cells

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Abstract

Tissue engineers and stem cell biologists have made exciting progress toward creating simplified models of human heart muscles or aligned monolayers to help bridge a longstanding gap between experimental animals and clinical trials. However, no existing human *in vitro* systems provide the direct measures of cardiac performance as a pump. Here, we developed a next-generation *in vitro* biomimetic model of pumping human heart chamber, and demonstrated its capability for pharmaceutical testing. From human pluripotent stem cell (hPSC)-derived ventricular cardiomyocytes (hvCM) embedded in collagen-based extracellular matrix hydrogel, we engineered a three-dimensional (3D) electro-mechanically coupled, fluid-ejecting miniature human ventricle-like cardiac organoid chamber (hvCOC). Structural characterization showed organized sarcomeres with myofibrillar microstructures. Transcript and RNA-seq analyses revealed upregulation of key Ca²⁺-handling, ion channel, and cardiac-specific proteins in hvCOC compared to lower-order 2D and 3D cultures of the same constituent cells. Clinically-important, physiologically complex contractile parameters such as ejection fraction, developed pressure, and

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Author contributions

T.J.C, W.K., K.D.C, R.A.L conceived the project, designed the experiments and prepared the manuscript. T.J.C, W.K., P.K.C., A.O.W, E.S.B and B.V.J. acquired and analyzed the mechanical and electrophysiological data. P.C.B. designed the hardware and wrote the software for data acquisition and analysis. K.D.C, C.W.C. and R.A.L supervised the experiments and provided the funding and overall guidance.

¹These authors contributed equally to this work.

Disclosure

R.A.L., C.W.Y.C., and K.D.C. hold equity in NovoHeart Holdings. Research conducted in this study could potentially affect the value of NovoHeart.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.biomaterials.2018.02.024>.

stroke work, as well as electrophysiological properties including action potential and conduction velocity were measured: hvCOC displayed key molecular and physiological characteristics of the native ventricle, and showed expected mechanical and electrophysiological responses to a range of pharmacological interventions (including positive and negative inotropes). We conclude that such “human-heart-in-a-jar” technology could facilitate the drug discovery process by providing human-specific preclinical data during early stage drug development.

Keywords

Cardiac tissue engineering; Human pluripotent stem cells; Ventricular pump function; Contractility; Electrophysiology

1. Introduction

Cardiac side effects have been a dominant reason for attrition of newly developed drugs, underscoring an urgent need for developing effective screening models that can help improve the success of costly clinical trials [1]. Human pluripotent stem cells (hPSC) can be specified into ventricular cardiomyocytes (vCM) that have been robustly characterized at the single-cell level [2–5]. Although hPSC-vCMs have been organized as monolayers and patches for electrophysiological assessment [6–8] and even surgical engraftment [9–11], such constructs are not designed for contractile characterization; by contrast, engineered cardiac thin films, micro-tissues and strips allow the assessment of twitch force [9,12–19], but not pressure- and volume-based metrics directly representing pump performance of the heart, and their physical forms limit utility for electrophysiological studies due to boundary effects. To advance the next generation of patient-specific *in vitro* cardiomimetic model systems, herein we describe the engineering of a 3D electro-mechanically coupled, fluid-ejecting human ventricle-like cardiac organoid chamber (hvCOC) that recapitulates key molecular, physiological and pharmacological features of the native ventricle. We demonstrated direct quantitative measurement of a variety of physiologically-integrated and clinically-familiar parameters such as ejection fraction, pressure-volume relationships, stroke work and electrophysiology all from a single engineered construct that ordinarily can only be monitored in whole hearts, opening new opportunities for basic and translational cardiac research applications using a pumping miniature human heart model.

2. Materials and methods

Experimental design.

A total of 25 hvCOC were fabricated and tested in order to characterize their structural, mechanical and electrophysiological functions. hvCOC were fabricated from hPSC-derived CMs. Only batches of hPSC-CMs with >70% of cells positively expressing cardiac troponin-T (cTnT) were included in the study. To study the response of hvCOC to various pharmacological agents, data from at least $n = 3$ hvCOC were collected for each intervention.

Human pluripotent stem cell culture and differentiation.

Ventricular (v) cardiomyocytes (CMs) generated from the pluripotent hES2 human (h) embryonic stem cell (ESC) line were used and generated as previously described [20].

Flow cytometry analysis. Efficiency of cardiac differentiation was determined by examining the expression of cTnT in each differentiation at day 20–25. In brief, cells were obtained from embryoid bodies (EBs) by enzymatically dissociating the EBs, fixing in 4% paraformaldehyde, permeabilizing and staining with anti-cardiac troponin-T before analyzing on a BD LSR II Flow Cytometer (BD Biosciences).

Human ventricular cardiac organoid chamber (hvCOC) generation.

After dissociation of EBs, the cell suspension was resuspended in high glucose DMEM (Sigma-Aldrich) containing 10% neonatal bovine serum (Atlanta Biologics), 1% penicillin-streptomycin (Corning) and 0.5% Amphotericin B (Sigma-Aldrich) (“NBS media”). The resuspended cells were then transferred to non-tissue culture treated dishes at a concentration of 2 million cells per 60 cm² for reaggregation. 48-hours later, the reaggregated cells were collected and resuspended in an ice-cold sterile solution containing type-I bovine collagen (Life Technologies) and hES-qualified Matrigel (Corning) to a final concentration of 2 mg/mL collagen, 0.9 mg/mL Matrigel and 10 million cells/mL tissue mixture of which about 90% were cTNT⁺MLC2v⁺ (Supplemental Fig. 1), with an additional 1 million cells/mL human dermal fibroblasts to aid in tissue compaction, similar to what we have previously described [14]. Briefly, the top of a 3 × 3 × 6 cm polystyrene bioreactor was modified to permit 9-gauge hypodermic tubing (Small Parts) to pass through the center and 4 mm diameter graphite electrodes (GraphiteStore) to flank the chamber on the edges of the bioreactor (Supplemental Fig. 2). A 6-Fr silicone balloon Foley catheter (Cook Medical) with the tip cut off and sealed with silicone caulking, was threaded through the 9-Gauge tubing into the center of the bioreactor. A small ring cut from hydrophilic porous polyethylene (Fisher Scientific) was placed just above the balloon on the hypodermic tubing. The balloon was filled with distilled water to the desired chamber size and positioned concentrically within a 2% agarose mold created in PBS and made by inserting a 13 mm diameter test tube (VWR) into the center of the agarose. The agarose mold forms the outer mold boundary while the catheter balloon forms the inner boundary (cf. Fig. 1a). The catheter was gently pulled against the hypodermic tubing and clamped above the tubing with a nylon screw compressor clamp (Fisher Scientific) to ensure the balloon did not move within the mold. 1 mL of the ice-cold sterile tissue mix was transferred to the mold, ensuring complete coverage of the porous polyethylene ring. The entire device was incubated for 2 h at 37 °C and 5% CO₂ to initiate gel polymerization, then immersed in NBS culture media. After 24 h, the top of the bioreactor, with the tubing, catheter and tissue, was removed from the agarose and transferred to a second polystyrene bioreactor containing 20 mL of NBS media. The tissue was maintained in this environment for 10 days at 37 °C and 5% CO₂, with daily half-media changes, during which time the tissue compacted around the balloon core to form a human engineered cardiac organoid.

Characterization of hvCOC contractile function.

After 10–12 days in culture, the silicone balloon was deflated and carefully removed from the inside of the organoid. A high-sensitivity pressure catheter (Millar) was advanced into the lumen of the hvCOC chamber and the catheter entry point was sealed. However, the pressure reservoir remained open to the atmosphere so that mean pressure load was controlled with an adjustable hydrostatic fluid column connected with flexible tubing to the 9 Ga hypodermic tube; the organoid was free to generate pressure when beating spontaneously or under electrical pacing (cf. Fig. 3a). A digital camera (PixelLINK) was mounted outside of the bioreactor and permitted direct tissue monitoring with images acquired at 43 frames/second. Chamber pressure and digital video were acquired simultaneously using a custom acquisition program built in LABVIEW (National Instruments) and chamber cross-sectional area was extracted from the video using a custom script in MATLAB (MathWorks). Chamber volume was estimated by assuming an equivalent sphere with the same cross-sectional area. Functional pump analysis was performed under field stimulation at 63 mV/mm with a GRASS S88x stimulator (Astro-Med) at specified frequencies. Where required, electrical field stimulation was induced with a pair of carbon electrodes 19 cm apart and a stimulation pulse length of 50 ms at 10 V. Both pressure and video signals were passed through a digital low pass filter with a 13-Hz cut-off frequency in MATLAB prior to data analysis. End-systolic pressure-volume relation (ESPVR) curves were fit to the maximal pressures and minimum volumes (end-systolic point) using a linear model, while end-diastolic pressure-volume relation (EDPVR) curves were fit to the maximal volumes and minimum pressures (end-diastolic point) using a nonlinear monoexponential regression of the form $EDP = Ae^{\beta \cdot EDV}$ where EDP is end-diastolic pressure, A is a curve fitting constant, β represents the diastolic stiffness constant and EDV is the estimated end-diastolic volume.

Characterization of hvCOC electrophysiological function.

For optical mapping of action potential, hvCOCs were incubated with a 1:1 ratio of a voltage-sensitive dye (di-8-ANEPPS, 10 μ M, Life Technologies) and Pluronic F-127 (20% solution, Life Technologies) for 2 h at 37 °C in NBS media. The staining solution was then removed and replaced by Tyrode's solution. A MiCAM Ultima-L Dual Camera System (SciMedia, USA), was used to measure dye fluorescence simultaneously on opposite sides of the chamber. The high-speed CMOS cameras have a 100 $\times$ 100 pixel resolution. Imaging was performed at 5 ms intervals. Excitation of the dye was completed using a halogen light source (HL-151, Moritex Schott, Japan) mounted with custom-made excitation/emission filters for di-4-ANEPPS (SciMedia, USA) that are also compatible with di-8-ANEPPS. Electrical field stimulation was completed in the same manner as when assessing contractile function. Alternatively for some electrophysiological studies, point electrical pacing was induced by a unipolar point electrode placed perpendicular to the surface of the hvCOC. Pacing was performed at the specified frequency (Hz) or cycle length (ms), with a 10 V stimulus and 10 ms duration. Throughout the optical mapping experiment, the silicone balloon inside the hvCOC remained inflated to impede spontaneous movement of the hvCOC to avoid motion artifacts. As a quality control measure, preparations demonstrating spontaneous reentrant arrhythmia in baseline condition (~20% of total preparations) were excluded from further analysis. Drugs were administered to the hvCOC via the media access

port cumulatively in a half log incremental manner. Imaging was performed 3 min after administration of each dose. Data analysis was completed with BrainVision software (SciMedia, USA). For optical mapping of intracellular calcium, hvCOCs were incubated with the calcium sensitive dye X-rhod-1 AM, 2.5 μ M (Life Technologies, USA) at 37 °C in NBS. Excitation of the dye was completed using a halogen light source (HL-151, Moritex Schott, Japan) mounted with excitation/emission filters for rhod 2 (SciMedia, USA).

Immunostaining.

hvCOC at day 10 after seeding were fixed with 4% paraformaldehyde in phosphate-buffered solution overnight and stained with primary and secondary antibodies before visualization via confocal laser scanning and multi-photon excitation microscopy.

Transmission Electron Microscopy.

The hvCOC was fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate-HCL buffer at pH 7.4 overnight at 4 °C and washed with cacodylate buffer. Post-fixation was done with 1% osmium tetroxide in cacodylate buffer for 1 h at room temperature and dehydrated using an ethanol series from 50% to 100%. hvCOC tissue was then infiltrated with epoxy resin/propylene oxide 1:1 mixture for 1.5 h at 37 °C followed by epoxy resin for 1 h at 37 °C and polymerized at 60 °C overnight. The fixed tissue was stained with 2% aqueous uranyl acetate and lead citrate. Images were taken with a Philips CM100 transmission electron microscope.

RNA extraction, cDNA synthesis, and gene expression analysis by real-time PCR.

Total RNA was extracted from samples of hvCOC at day 10 after seeding using RNeasy Mini Kit (Qiagen) following DNase I (Promega) treatment for the removal of potential contamination of genomic DNA. cDNA were prepared using the QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's protocol. Gene expression levels were quantified using StepOnePlus™ Real-Time PCR system (Applied Biosystems).

Sample preparation and processing for RNA sequencing analysis.

RNA samples of age-matched single hPSC-CMs, hvCMT [18], hvCTS fabricated with the same batches of differentiated hPSC-CMs were harvested and extracted by Trizol. DNA libraries were prepared by KAPA Stranded mRNA-Seq Kit (Kapa Biosystems) (n = 2). HiSeq PE Cluster Kit v4 with cbot and HiSeq SBS Kit v4 (Illumina) was used for Pair-End 101bp sequencing by HiSeq 1500 (Illumina). Manufacturers' protocols were followed. Sequencing reads were first filtered for adapter sequence and low quality sequence followed by retaining only reads with read length $\geq$ 40bp. The rRNA-filtered raw reads were aligned to human reference genome GRCh37 using TopHat, and transcriptome assembly made using Cufflink. For each collection of gene sets, the gene sets with FDR-corrected p-value less than 0.20 were deemed significantly varied (enriched or depleted) ones. Gene Ontology (GO) Enrichment Analysis and KEGG pathway Enrichment Analysis were performed with the online web-based application 'WEB-based GEne SeT AnaLysis Toolkit' (<http://www.webgestalt.org>).

Statistical Analysis.

Statistical analysis was performed using GraphPad Prism. Descriptive statistics are reported as the mean and standard error of the mean (SEM) of multiple hvCOC, unless otherwise specified. Differences between baseline and pharmacological treatment were analyzed via paired Student's t-test between baseline and treatment of the same hvCOC or, where multiple concentrations were applied, one-way ANOVA with post-hoc pairwise comparisons utilizing the Bonferroni correction. Linear regression was used to determine the significance of the negative force-ejection fraction relation. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Bioengineering hvCOC

First, we developed a custom bioreactor (Fig. 1a–e, Supplemental Fig. 2) to construct hvCOCs resembling a simplified heart pump; this device aimed to enhance reproducibility and efficiency by enabling subsequent mechano-electrophysiological characterization of the constructed hvCOC to be performed within the same bioreactor. To form hvCOC, a cell suspension of $\sim 10^7$ hPSC-vCMs ($\sim 90\%$ cTNT⁺MLC2v⁺) were mixed with a small number of non-cardiomyocytes ($\sim 10\%$ dermal fibroblast) and aqueous scaffold materials in our bioreactor, followed by gelation and self-assembly during incubation for 10 days (Supplemental Fig. 2). Following incubation for 10 days, the resulting hvCOCs exhibited a hollow chamber geometry (Fig. 1f) with cells distributed across a wall thickness of approximately 100 μm (Fig. 1g). Immunostaining showed positive expression of α -actinin and the mitochondrial protein COX IV (Fig. 1h), with sarcomeric arrangements similar to other human engineered cardiac tissue constructs [14,21–23] but distinct from random clusters. Electron microscopy further revealed clearly visible Z-lines and other sub-sarcomeric structures and myofibrils intercalated with mitochondria (Fig. 1i), with a sarcomere length of about 1.6 μm due to partial contracture of the tissue. Cell junctions including desmosomes and gap junctions were also identified (Supplemental Fig. 3). Transcript analysis of 2D-cultured clusters [24] and alternative engineered constructs, including human ventricular cardiac micro-tissues (hvCMT; ~ 1000 cells) [18], tissue strips (hvCTS; $\sim 10^6$ cells) [14] and hvCOC made of identical constituent hPSC-vCMs, revealed changes consistent with our previous maturation studies [18,24–28]. As shown in Fig. 2a, several key Ca^{2+} -handling transcripts such as PLN and RYR that are lowly expressed in hPSC-CMs became significantly upregulated in hvCOC. Electrophysiologically, Kir2.1 (KCNJ2), which mediates the inwardly rectifying potassium current important for electrical stability of hPSC-CMs [5,29], was upregulated in hvCOC by up to about 5-fold. Cardiac contractile transcripts MYH6 and MYH7 were upregulated by > 20 -fold. Connexin-43 was elevated in hvCOC compared to the other engineered constructs, though not different from 2D culture. Based on these observations using identical cardiomyocyte sources within distinct tissue configurations, we hypothesized that hvCOC provides a unique environment with non-cell autonomous cues that promote maturation, presumably due to the cardiomimetic 3D chamber geometry, pumping action-related mechanical load and other biomimetic stimuli. Venn diagrams of differentially expressed genes (DEG) by RNA-sequencing (Fig. 2b) indicate the greatest homology between hvCOC and hvCTS;

nevertheless, the largest number of tissue-specific DEGs belong to hvCOC, supporting the concept that this uniquely cardiomimetic culture environment gives rise to cells expressing a distinct molecular signature. Accordingly, principal-component analysis of the global transcriptomic signatures showed hvCOC positioned on one extreme, followed by lower-ordered hvCTS and hvCMT, with randomly oriented 2D hPSC-vCM clusters on the opposite extreme (Fig. 2b). When the differentially regulated transcripts were examined and ranked by the percent of genes changed in their corresponding biological pathways, adrenergic signaling, cGMP-PKG signaling and Ca^{2+} signaling stood out among the top four upregulated pathways in hvCOC (see also Supplemental Fig. 4a).

3.2. Pump performance of hvCOC and their responses to pharmacological interventions

Next, we functionally characterized the contractile properties of hvCOCs using a custom-designed fluid system to control loading pressure and measure the oscillatory pressures generated inside the lumen of the beating organoid (Fig. 3a). Mechanically, cyclical changes in pressure (P) and volume (V) were out-of-phase such that an increase in chamber pressure was accompanied by a decrease in volume, and vice versa; as a result, the reconstructed PV loops exhibited a counter-clockwise progression throughout the beating cycle (see Fig. 3b–c for a representative experiment). Further detailed analysis revealed the following baseline properties averaged from 25 hvCOCs (Fig. 3d): spontaneous beating frequency, 0.76 ± 0.06 Hz; developed (max-min) pressure, 1.26 ± 0.12 mmH₂O; stroke volume, 4.82 ± 0.63 μl ; stroke work, 5.832 ± 1.267 mmH₂O μl ; ejection fraction, $2.44 \pm 0.27\%$; cardiac output, 212.9 ± 28.6 $\mu\text{l}/\text{min}$. Upon electrical pacing, hvCOC synchronously responded to a physiological range of frequencies from 0.5 to 3.0 Hz (Fig. 3e–f; Movie S1). Developed pressure exhibited a negative pressure-frequency relationship (Fig. 3f), similar to the negative Ca^{2+} transient-frequency data in hvCMs [24] and negative force-frequency relationships observed for hvCMT [18], hvCTS [14] and newborn human heart muscle [30], as commonly seen with engineered heart tissues. A number of groups have reported engineered tissues with contractile forces and conduction properties approaching physiologic levels observed in native myocardium [8,13,31,32]. However, in contrast to constructs designed for surgical implantation [8], engineered tissues designed for *in vitro* applications, such as our organoids, may not require physiologic levels of function to be predictive and impactful, provided they can recapitulate cardiac-appropriate responses to treatments and interventions of interest. Prior cardiac tissue engineering studies have shown this in the context of arrhythmogenicity [33], contractility [14,34] and recapitulation of disease phenotypes [12,48]. This was further tested in the experiments that follow.

Supplementary video related to this article can be found at <https://doi.org/10.1016/j.biomaterials.2018.02.024>.

A hallmark characteristic of cardiac physiology is the Frank-Starling mechanism by which the ventricular stroke volume increases as the volume of filling blood increases. In native cardiac muscle experiments, this translates into the generation of stronger twitch forces with increasing preload or stretch [35]. Similarly, we have reported a linear relationship between the developed stress and axial stretch in hvCTS [14]. Consistent with a functional Frank-Starling mechanism [36] and similar to the load-dependent contractile reserve in native

myocardium, a linear relationship between developed pressure and the mean pressure load was observed in hvCOCs pumping at a controlled pacing frequency of 1 Hz ($R^2 = 0.99$, Fig. 3g). At selected loading pressures the reconstructed P-V loops progressed counter-clockwise (Fig. 3h), indicating positive stroke work that increased with mean pressure. The average maximum pressure points followed a linear end-systolic pressure-volume relation (ESPVR) ($R^2 = 0.98$) with a slope of $0.28 \text{ mmHg} \cdot \text{mm}^{-3}$ representing the end-systolic elastance (E_{es}), a clinically valuable index of ventricular contractility. An exponential regression fit the average minimum pressure points ($R^2 = 0.99$) yielding an end-diastolic pressure-volume relation (EDPVR) with a diastolic stiffness constant of $0.012 \text{ } \mu\text{l}^{-1}$ reflecting the passive compliance of the hvCOC. As anticipated for an operating ventricular preparation, the developed pressure increased with increasing external Ca^{2+} concentration, demonstrating positive inotropy (Fig. 3i). To our knowledge, these hvCOCs represent the first human engineered cardiac tissue constructs that allow direct measurement of multiple metrics of cardiac pump performance that are of primary interest in cardiology.

Pharmacologically, 2D-cultured hPSC-vCMs [18], as well as 3D hvCMTs [21] and hvCTSs [14] created from such cells, have been reported to display positive chronotropic but weak or even null inotropic responses to β -adrenergic stimulation (Supplemental Fig. 5). The weak response is thought to be due to immature adrenergic and Ca^{2+} signaling pathways such as lowered phospholamban expression compared to their adult counterparts [14]. Guided by our RNA-seq and qPCR data, we postulated that hvCOC would exhibit more mature responses to β agonists. When exposed to isoproterenol ($n = 5$), the spontaneous beating rate of hvCOCs significantly increased by $>200\%$ at $1 \text{ } \mu\text{M}$, ($p < 0.05$; Fig. 4a), consistent with a positive chronotropic response. When isoproterenol ($1 \text{ } \mu\text{M}$)-treated hvCOCs were paced at 1 Hz (so as to control frequency-dependent effects on contractility), the developed pressure, stroke volume, and stroke work likewise significantly increased by approximately 25–50%, ($p < 0.05$) indicating a positive inotropic effect. When we examined the dose-response relationship for the calculated cardiac output with both chronotropic and inotropic responses taken into consideration, the half maximal effective concentration (EC_{50}) was $231.7 \pm 16.8 \text{ nM}$ (Supplemental Fig. 6), comparable to a value of 800 nM tested in human non-failing heart muscle [37]. In addition, the representative P-V loop showed an increase in peak systolic pressure and stroke work with isoproterenol, consistent with a positive inotropic effect (Fig. 4b). Digoxin ($n = 5$), another positive inotrope, significantly increased pressure, stroke volume, stroke work and cardiac output as well as the ejection fraction without significantly affecting the frequency at $0.1 \text{ } \mu\text{M}$ (Fig. 4a–b). Cardiac output-based EC_{50} was $34.9 \pm 26.3 \text{ nM}$ for digoxin treatment. Conversely, organoid pump performance was significantly attenuated when treated with the well-defined negative inotropes verapamil ($n = 3$), nifedipine ($n = 3$) and disopyramide ($n = 3$) (Fig. 4a). The corresponding P-V loops (Fig. 4b) were in complete accordance with these changes. The EC_{50} values for their cardiac outputs were $61.7 \pm 6.6 \text{ nM}$, $15.4 \pm 9.4 \text{ nM}$ and $354.5 \pm 42.7 \text{ nM}$, respectively, which are in the range of those previously reported with Langendorff heart experiments [38,39].

3.3. Electrophysiological function of hvCOC

For a more comprehensive characterization, we also performed electrophysiological mapping of hvCOC to investigate the electrical conduction and action potential (AP)

properties. An isochronal conduction map of the hvCOC surface by high-resolution optical mapping during point stimulation revealed systematic propagation of depolarizing signals originating from the pacing site (Fig. 5a). Baseline AP parameters are summarized in Fig. 5b. As the pacing frequency was increased (Fig. 5c) (i.e., shorter basic cycle length, BCL), the AP duration at 50% amplitude (APD₅₀) (Fig. 5d) and the conduction velocity (CV) (Fig. 5e) decreased, indicating the ability of hvCOC to rate-adapt, another key physiological feature of native cardiac cells and tissues [40,41]. Furthermore, Fig. 5c shows the immediate initiation of an AP (black tracing) upon 1-Hz electrical stimulation (black arrows at 0 and 1000 ms) followed by Ca²⁺ transient initiation (blue tracing) after a systematic and reproducible delay, as anticipated from Ca²⁺-induced-Ca²⁺-release upon Ca²⁺ influx via L-type Ca²⁺ channels [28] that subsequently lead to cardiac contractions.

The effects of drug interventions on electrophysiological properties of hvCOC were also examined. As described in guinea pig ventricular myocytes [42], the L-type Ca²⁺ channel blocker verapamil initially increased and then at higher concentrations decreased the APD₅₀, with a shortened plateau phase and reduced amplitudes, thereby secondarily increasing the firing frequency (Fig. 6a, d). Consistent with a negative inotropic effect resulting from reduced Ca²⁺ entry that we reported in our previous studies with hvCTS and hvCMT [14,18], the stroke volume, cardiac output, and developed pressure decreased (shown in Fig. 4a) with the P-V loops likewise exhibiting decreases in end-systolic pressure and stroke work (Fig. 4b). Contractions and developed pressures were completely abolished at 10 μ M verapamil. Taken together, these data demonstrate functional electro-mechanical coupling of hvCOC. As for positive inotropic interventions, the addition of isoproterenol dose-dependently increased spontaneous AP firing with concomitant increases in CV and decreases in APD₅₀ (Fig. 6a and b), consistent with an intact chronotropic response and mirroring the increased spontaneous beating rate already presented in Fig. 4a. In stark contrast, the voltage-gated Na⁺ channel blocker flecainide potently and dose-dependently reduced the firing frequency and CV primarily by prolonging APD₅₀ as a result of decreased upstroke velocity and reduced amplitudes (Fig. 6a, c). At 30 μ M flecainide, all electrical activity was abolished.

4. Discussion

In this study, we report a new platform, namely hvCOC, that allows clinically relevant and physiologically complex parameters such as pressure-volume relationships, ejection fraction and cardiac output to be measured from a single engineered construct. The hvCOC system is complementary to other existing tools for drug discovery and disease modeling, and allows organ-level measurements of cardiac pump performance that have not been previously possible with human *in vitro* systems.

Herein, the same constituent hPSC-vCMs, whose single-cell properties (e.g., EP, Ca²⁺, transcriptomes, miRNA-omes, proteomes) have been extensively characterized by us and others, were reassembled in different engineered constructs for direct comparison so as to reveal aspects unique to hvCOC. The improved functionality of the hvCOC was compared to other 3D [14,18] and 2D [43] models. Functionally, we observed an augmented sensitivity of the hvCOC to adrenergic stimulation and the presence of both chronotropic and inotropic

effects when compared with the hvCTS and hvCMT systems constructed with the same hPSC-vCMs [14,18]. This observation was consistent with an upregulation of PLB as demonstrated by our qPCR and the ranking of the pathways ‘cGMP-PKG signaling pathway’ and ‘calcium signaling pathway’ among the top 10 pathways with the highest number of upregulated genes in KEGG pathway analysis of the RNA-sequencing data (Supplemental Fig. 4a). The pathway with the highest enrichment score was ‘Adrenergic signaling in cardiomyocytes’ (Supplemental Fig. 4b). Gene Ontology (GO) Enrichment Analysis also revealed that the top 10 GO terms in Biological Processes that are over-represented are all related to the cardiovascular system and muscle biology. Similarly, 9 out of the 10 top over-represented GO terms in Cellular Components are related to cardiomyocytes (Supplemental Fig. 4c). In addition, consistent with the observed increase in expression of cardiac proteins including MHC α (MYH6) and MHC β (MYH7) with qPCR, RNA-sequencing data also showed increases in the expression of cardiac contractile proteins including myosin, cardiac troponin T and cardiac troponin I. Of note, while improved maturation was observed, the hvCOC model is not intended to achieve the physiologic levels of function observed in adult hearts. Nevertheless, our experimental design for testing hvCOC responses consistently parallels the responses expected in native heart function, as demonstrated using five drugs with known inotropic effects.

Although we and others previously reported the construction of heart chambers from rodent cells [44,45], advancing the technology to human cells faced numerous significant technical challenges that have now been overcome with our improved cell differentiation protocol and custom bioreactor, such that operator manipulations are minimized after assembly for subsequent phenotypic measurements, leading to substantially improved success rate, throughput and resolution; in particular, our bioreactor design enables a single researcher to assemble 8–10 hvCOCs per week with a success rate of about 70–80%. For the first time, clinically-meaningful parameters such as ejection fraction, pressure-volume relationships, cardiac output, stroke work, EDPVR, ESPVR, PV loops as well as electrophysiological mapping under multiple loading conditions or drug interventions could now be measured from a single human cardiac tissue construct.

The signal-to-noise ratio due to small EFs could limit the sensitivity of hvCOC-based assays. This small EF was primarily attributed to the thin wall (relative to the diameter, indicating high wall stress) and other contributing factors including random cell alignment and limited cellular maturity common in most other stem-cell derived systems. Indeed, approximately 50% increase in pressure and ejection fraction and 100% increase in stroke work could be achieved by prolonging the culture time to Day 20 (data not shown). Achieving large EF likely requires the specialized multi-layered helically aligned hierarchical muscle fiber organization of the native adult heart, with integrated vasculature to support viability of the thick-walled tissue. Our objective here was to provide a simplified *in vitro* model, that is amenable to analysis based on equations like the Law of Laplace, that allows independent control of chamber size and cell type, that captures the essential features of native ventricular pump function, and that can be scaled up for robust and reliable *in vitro* screening. Considering these simplifications, it is noteworthy that in the day 10.5e11 embryonic mouse LV, in which the heart muscle also has a random organization, the reported EF is only about 27% [46], which may be a more appropriate comparison for our

hvCOC than an adult EF of 70%. Despite the sub-physiologic function, the hvCOC system was able to distinguish dose responses of positive and negative inotropes. Finally, because the size of the hvCOC is significantly smaller than the native human heart and closer to the size of a mouse heart, it may have limitations for modeling sustained reentrant arrhythmia and fibrillation, which are known to be less prevalent in rodents [47]. However, the propensity for arrhythmia formation is also related to functional and/or structural heterogeneities that can cause variable conduction velocities across a cardiac surface, which can be detected by optical mapping as locally hastened or slowed CVs. Indeed, the roughly 4-fold increase and 5-fold decrease in CV of hvCOC after isoproterenol and flecainide treatments, respectively, suggests such effects can be readily captured in our system.

To conclude, the fluid-ejecting hvCOC system recapitulates critical physiological and pharmacological features of the native human heart. The generation of physiological parameters extends the measurements of contractility and electrophysiology to the organ level, unprecedented by any existing human *in vitro* systems. Combined, the ability to measure both mechanical and electrical properties in a simplified yet biomimetic model of a human ventricle offers new avenues to help bridge the longstanding gap between traditional cell culture systems, *in vivo* animal models and clinical trials. In combination with somatic reprogramming of induced hPSC and the control of cellular composition as well as the external milieu, such a “human-heart-in-a-jar” system offers an advanced biomaterial platform representing the upper end of a tiered screening approach for next-generation drug discovery, cardiotoxicity screening, disease modeling and other ethnicity-, sex- and patient-specific applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Engineering human ventricular cardiac organoid chambers (hvCOC).

Schematics of (a) hvCOC construction in our custom-designed bioreactor during the first 24 h, and (b) after removal of the outer agarose mold. (c, d) The corresponding images. (e) The entire hvCOC bioreactor with carbon rods in place for electrical field stimulation. (f) A representative image of a contracting hvCOC (see Movie S1). (g) Hematoxylin-eosin stained full-thickness transverse section of hvCOC wall. (h) Immunostaining for α -actinin (red) and Cox-IV (green) with DAPI-stained nuclei (blue). (i) Transmission electron micrographs. Myo, myofilaments; Z, Z-line; I, I-band; A, A-band; H, H-zone; mito, mitochondria. Each image is taken from a separate hvCOC.

a Calcium handling proteins

Ion channel proteins

Cardiac proteins

b Upregulated genes Downregulated genes

Fig. 2. Transcriptomic analysis of human ventricular cardiac organoid chambers (hvCOC).

(a) RT-PCR of various key cardiac transcripts in hvCOC, including calcium handling proteins (*top*), ion channel proteins (*middle*) and cardiac-specific proteins (bottom), compared to lower ordered 2D-cultured random clusters (2D-hvCMs), microtissues (hvCMT), and tissue strips (hvCTS) made from identical hPSC-vCMs. Error bars represent standard error of the mean (n = 3 cultures per group) *p < 0.05 vs. 2D-hvCMs. (b) *Left*: Venn diagrams showing differentially regulated transcripts by RNA-seq of hvCMT, hvCTS and hvCOC normalized to those of 2D-hvCMs. *Right*: Principal-component analysis.

Fig. 3. Mechanobiological characterization of hvCOC.

(a) Schematic of the pressure-volume testing system. The height of the fluid reservoir can be adjusted to control the hydrostatic pressure load on the hvCOC. The resulting pressure within the hvCOC lumen was recorded via a pressure catheter synchronized with a high-speed video camera for monitoring changes in chamber size. (b) Changes in hvCOC volume were associated with out-of-phase pressure pulses. (c) The relationship between pressure and volume produced counterclockwise loops indicative of positive stroke work performed by the actively contracting hvCOC. (d) Baseline parameters measured from hvCOCs constructed from hPSC-CMs including beating frequency, developed pressure, stroke volume, stroke work, ejection fraction, and cardiac output ($n = 25$). Bars represent mean $\pm$ SEM. (e) Raw tracings of the cross-sectional area versus time during pacing with electrical field stimulation at 0.5 and 2.0 Hz. (f) Developed pressure decreased with increasing pacing frequency. Error bars represents standard error of the mean of multiple contractions of a representative experiment. (g) At a representative pacing frequency of 1.0 Hz, the developed pressure increased linearly as the mean pressure load was elevated. (h) End-systolic pressure-volume relationship (ESPVR) and end-diastolic pressure-volume relationship (EDPVR). (i) Increasing developed pressure was observed with increasing external $[Ca^{2+}]$, $n = 3$. Error bars represent standard error of the mean of multiple contractions.

Fig. 4. Mechanical function of hvCOC in response to pharmacological intervention.

(a) Changes in measured parameters upon administration of pharmacological agents including isoproterenol (iso, 1 μ M, n = 5 hvCOC), digoxin (dig 0.1 μ M, n = 5 hvCOC), verapamil (ver, 1 μ M, n = 3 hvCOC), nifedipine (nif, 1 μ M, n = 3 hvCOC) and disopyramide (diso, 1 μ M, n = 3 hvCOC). All parameters were measured at 1 Hz pacing except frequency and cardiac output, which were obtained during spontaneous beating. Error bars represent standard error of the mean of multiple hvCOC indicated by the sample size, n, above. * $p < 0.05$ vs. baseline of hvCOC constructed from hPSC-CMs. (b) Example normalized P-V loops, each representing one hvCOC at drug-free baseline and upon drug administration, showing clear effects of each intervention on developed pressure and stroke volume.

Fig. 5. Electrophysiological characterization of hvCOC.

(a) Representative isochronal conduction map, overlaid on a photograph of the hvCOC. Circle indicates electrical lead position for point-stimulation. The blank region at the top of the isochronal map represents an area near the porous ring of the hvCOC where there was very low signal due to the locally low concentration of hPSCeCMs. (b) Electrophysiological parameters of hvCOC constructed from hPSC-CMs including AP firing frequency, upstroke time, APD₅₀, APD₉₀, and conduction velocity. (c) *Left*: Representative tracing showing AP firing at various basic cycle lengths, (BCL). *Right*: Overlapped AP (black) and Ca²⁺ (blue)

transient tracings revealed a systematic delay as a result of EC coupling. **(d)** APD_{50} and **(e)** conduction velocity decreased with increasing pacing frequency (i.e., decreasing BCL). Error bars represent standard error of the mean of APs from multiple experiments. $n = 3$.

Fig. 6. Electrophysiological function of hvCOC in response to pharmacological intervention.

(a) Representative experiments showing tracings of optical mapping of hvCOC after administration of isoproterenol, flecainide and verapamil. (b) Isoproterenol dose-dependently and chronotropically increased the spontaneous firing frequency with concomitant decreases in APD₅₀ and increases in conduction velocity. (c) The Na⁺ channel blocker flecainide dose dependently decreased AP amplitude and slowed upstrokes, resulting in a slowing of firing frequency, prolongation of APD₅₀ and decrease in conduction velocity. (d) Administration of the L-type calcium channel blocker verapamil decreased the AP amplitude with an associated decrease in APD₅₀ and increase in firing frequency at higher dosages. CV = Conduction velocity; APD₅₀ = action potential duration at 50% of

repolarization. Error bars represent standard error of the mean of APs from multiple experiments. * $p < 0.05$, $n = 3$.