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Tissue-engineered Cardiac Patch for Advanced Functional Maturation of Human ESC-derived Cardiomyocytes

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Abstract

Human embryonic stem cell-derived cardiomyocytes (hESC-CMs) provide a promising source for cell therapy and drug screening. Several high-yield protocols exist for hESC-CM production; however, methods to significantly advance hESC-CM maturation are still lacking. Building on our previous experience with mouse ESC-CMs, we investigated the effects of 3-dimensional (3D) tissue-engineered culture environment and cardiomyocyte purity on structural and functional maturation of hESC-CMs. 2D monolayer and 3D fibrin-based cardiac patch cultures were generated using dissociated cells from differentiated Hes2 embryoid bodies containing varying percentage (48-90%) of CD172a (SIRPA)-positive cardiomyocytes. hESC-CMs within the patch were aligned uniformly by locally controlling the direction of passive tension. Compared to hESC-CMs in age (2 weeks) and purity (48-65%) matched 2D monolayers, hESC-CMs in 3D patches exhibited significantly higher conduction velocities (CVs), longer sarcomeres (2.09 ± 0.02 vs. 1.77 ± 0.01 μm), and enhanced expression of genes involved in cardiac contractile function, including cTnT, αMHC , CASQ2 and SERCA2. The CVs in cardiac patches increased with cardiomyocyte purity, reaching 25.1 cm/s in patches constructed with 90% hESC-CMs. Maximum contractile force amplitudes and active stresses of cardiac patches averaged to 3.0 ± 1.1 mN and 11.8 ± 4.5 mN/mm², respectively. Moreover, contractile force per input cardiomyocyte averaged to 5.7 ± 1.1 nN/cell and showed a negative correlation with hESC-CM purity. Finally, patches exhibited significant positive inotropy with isoproterenol administration (1.7 ± 0.3 -fold force increase, $\text{EC}_{50} = 95.1$ nM). These results demonstrate highly advanced levels of hESC-CM maturation after 2 weeks of 3D cardiac patch culture and carry important implications for future drug development and cell therapy studies.

Keywords

Human pluripotent stem cells; hydrogel; optical mapping; adrenergic stimulation; cardiac tissue engineering

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Additional details and assessment methodologies are provided in Supplemental Materials.

Introduction

Since the discovery of human embryonic (hESCs) and induced pluripotent stem cells (hiPSCs), significant efforts have been made to enable efficient production of human cardiomyocytes (CMs) [1]. Of particular importance to clinical translation are the recently developed techniques for high-yield cardiac differentiation that do not require the use of genetic modifications [2-7]. Furthermore, new purification protocols based on cell-surface markers (SIRPA, VCAM1) [8, 9], mitochondrial fluorescence dyes (TMRM) [10], or distinct metabolic flows in CMs and non-CMs [11] have enabled generation of virtually pure (>95%) cardiomyocyte populations. Despite these advances, human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) retain a relatively immature phenotype and, unlike their adult counterparts, exhibit relatively small size, reduced electrical excitability [1, 12], impaired excitation-contraction coupling [13-17] and incomplete adrenergic sensitivity [18, 19]. The lack of robust methodologies to promote functional maturation of human cardiomyocytes is currently one of the critical obstacles to the successful development of predictive drug and toxicology screens as well as safe and efficient cardiac therapies.

In general, functional maturity of cardiomyocytes at the tissue level is evidenced by their ability to support fast action potential conduction and generate high contractile stresses. Previously, genetically purified (>95%) hiPSC-CMs were shown to support relatively fast (~21 cm/s) electrical conduction in 2D cultures (monolayers) [20], although similar velocities have not been achieved in a handful of studies that assessed the functional output of engineered 3D human heart tissues [19, 21-23]. In the best reported case [21], tissues made of genetically purified hESC-CMs exhibited contractile stresses (4.4 mN/mm²) and conduction velocities (<4.9 cm/s), an order of magnitude lower than adult human myocardium (20-45 mN/mm² and 40-50 cm/s) [24, 25]. As such, it remains unknown whether hPSC-CMs can be matured *in vitro* to attain both high CVs and contractile stresses, as well as how cardiomyocyte purity and 3D culture affect functional output and maturation of human engineered cardiac tissues.

To address these questions, we generated 2D and 3D cultures of human ESC-CMs based on our recent experience engineering functional cardiac tissues using mouse ESC-CMs [26]. The goal of 3D culture model was to assess the electrical and mechanical maturation of hESC-CM. Specifically, quantification of electrical impulse propagation and mechanical force generation was used to assess the overall tissue function, with subsequent immunofluorescence and genetic profiling to assess the structural and molecular properties of the hESC-CMs. Finally, levels of advanced maturity were assessed using beta-adrenergic stimulation of the engineered tissues.

Materials and Methods

Cardiac differentiation of hESCs

Human ES cells (HES-2 line) were trypsin-adapted and differentiated to a cardiovascular lineage based on previously described methods (Fig. S1A) [9]. Briefly, two days prior to differentiation (d-2), hESCs were plated on Matrigel (BD Biosciences) coated plates for feeder depletion. EBs were formed by aggregating trypsinized ES cell clusters in StemPro-34 media containing BMP4 (0.5 ng/ml) in low-attachment plates overnight (d-1) in humidified incubator and cultured under hypoxic conditions (5% O₂, 5% CO₂, 37°C) until day 11. On day 0 (start of differentiation), EBs were harvested and resuspended in Induction Medium (StemPro-34, bFGF (5 ng/ml), activin A (6 ng/ml) and BMP4 (10 ng/ml)) and cultured for 3 days. On day 4, EBs were harvested and resuspended in StemPro-34 supplemented with VEGF (10 ng/ml) and Wnt-C59 (2 μM, Cellagen Technology). Medium was exchanged on day 6 with StemPro-34 supplemented with VEGF (10 ng/ml). On day 8,

11, and 15, medium was exchanged with StemPro-34 supplemented with only VEGF (10 ng/ml) and bFGF (5 ng/ml). On day 11, EBs were moved from hypoxic (5% O₂, 5% CO₂, 37°C) to ambient oxygen condition (5% CO₂, ambient air, 37°C). From day 18 on, medium was replaced with fresh 2% FBS/DMEM every 3 days. hESC-CMs were used on days 22-30 of differentiation. All StemPro-34 media included L-glutamine (2 mM), transferrin (150 µg/ml, Roche), ascorbic acid (50 µg/ml), and monothioglycerol (0.45 mM). Medium supplements were purchased from Life Technologies Corporations, unless otherwise stated. Chemicals were obtained from Sigma Aldrich and all growth factors were obtained from R&D Systems.

Dissociation of differentiated cell clusters

Beating cell clusters were dissociated using 0.2% collagenase type I (Sigma C-0130) in 20% FBS/DMEM (Gibco) at 37°C for 1 hour, followed by 0.25% trypsin/EDTA (Cellgro) with gentle shaking in a 37°C water bath for 5-7 minutes. Single-cell suspension was achieved by triturating with equal volume of 50% FBS/DMEM + 20 µg/ml DNase (Calbiochem).

Purification of hESC-CMs

For monolayer and patch cultures, the fraction of cardiomyocytes in dissociated cells was enriched by magnetic-activated cell sorting (MACS). Briefly, dissociated cells (5×10⁶ cells/ml) were stained with PE-Cy7 conjugated anti-SIRPA-IgG antibody (clone SE5A5; BioLegend; 1:500). Labeled cells were loaded with anti-PE-Cy7 MicroBeads, and the cell/bead mix was passed through MS Columns on a MiniMACS™ separator (Miltenyi Biotec). Small fractions of immunolabeled cells (MSC sorted and unsorted) were then analyzed using the flow cytometer FACSARIA II with FACSDiva software, version 6.0 (BD Biosciences). Cardiac specificity of SIRPA was confirmed by FACS analysis (Fig. S1B-D) for cardiac troponin T (cTnT) which showed that >92% of SIRPA⁺ cells in MACS sorted and unsorted populations consisted of cTnT⁺ cardiomyocytes. Based on this analysis, MACS sorted and unsorted cells were mixed in different ratios to obtain 48-90% pure hESC-CM populations used for patch and monolayer production.

Cardiac patch fabrication and culture

To generate aligned 3D human cardiac tissue patches, 7×7mm² polydimethylsiloxane (PDMS, Dow Corning) molds with staggered hexagonal posts (1.2 mm long) were microfabricated as previously described [27]. Hydrogel solution (24 µL fibrinogen (10 mg/mL), 12 µL Matrigel, 24 µL 2x cardiac media)) was mixed with 1×10⁶ cells in 59 µL cardiac media to obtain a total of 120 µL of cell/gel solution. Following addition of thrombin (0.92 µL), cell/gel solution was added to PDMS molds containing a Velcro frame and left at 37°C for 1hr to polymerize. Resulting cardiac patches were cultured with rocking in cardiac medium (5% FBS/DMEM with 1mM sodium pyruvate (Gibco), 2mM Glutamine (Gibco), 0.1mM non-essential amino acids (Gibco), 50 µg/ml Ascorbic Acid (Sigma), and 0.45mM monothioglycerol (Sigma)) for 4-14 days. Culture media was supplemented daily with 1mg/mL aminocaproic acid (Sigma) to prevent fibrin degradation. 10µM BrdU (Sigma) was added on day 0 of patch culture to inhibit proliferation of non-myocytes and preserve initial purity of hESC-CMs. BrdU was removed after 24 h of culture, and media was changed every 2 days thereafter.

Assessment of electrical propagation

Optical mapping of transmembrane potentials was performed after 2 weeks of culture using our established methods [26, 28, 29]. Two-second episodes of electrical activity induced by stimulation with point electrode were recorded in macroscopic (whole tissue) or microscopic (4x objective on a Nikon microscope) mode using a 504-channel photodiode array (RedShirt

Imaging) or a fast EMCCD camera (iXon^{EM+}, Andor). Data analysis was performed using custom MATLAB software [28].

Assessment of contractile force generation

Force generating capacity of cardiac tissue patches was assessed in 2-week old patches loaded into a custom-made isometric force measurement setup containing a sensitive optical force transducer and a computer-controlled linear actuator (Thorlabs), as previously described [26, 29, 30]. Inotropic responsiveness of the tissue patches was tested by measurement of contractile force generation in the presence of 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} and 10^{-5} M β -adrenergic agonist isoproterenol in 0.9mM Ca^{2+} Tyrode's solution during 1Hz electrical stimulation at 10% stretch.

Statistical analysis

Experimental data reported as mean $\pm$ SEM was compared by one-way ANOVA and unpaired t-test. Wilcoxon Rank-Sum test (median, Z-score, p-value) was used for non-normal distributions. A bivariate linear regression analysis was performed to determine significance of linear fits. A p-value of 0.05 was considered significant.

Results

Electrophysiological properties of hESC-CMs

Action potential properties of dissociated unpurified hESC-CMs were assessed using patch-clamp recordings. After 20-30 days of differentiation cardiomyocytes exhibited a predominantly ventricular action potential (AP) phenotype with hyperpolarized resting membrane potentials (-70.9 ± 0.5 mV) and relatively fast maximum upstroke velocities (38.1 ± 1.5 V/s) (Fig. S2A). Studied hESC-CMs exhibited expected dose-dependent responses to HERG K^+ channel blocker E-4031, L-type Ca^{2+} channel blocker nifedipine, and ATP-sensitive K^+ channel blocker terfenadine (Fig. S2B). Specifically, the application of E-4031 and terfenadine caused a significant increase in action potential duration at 90% repolarization (APD90), while nifedipine caused a large decrease in APD90 (Fig. S2C), showing physiological responses to inhibition of K^+ and Ca^{2+} channels.

Structural phenotype of human cardiac tissue patches and monolayers

Differentiated cell populations with varying hESC-CM purity (48-90%) were cultured in 20 mm-diameter confluent monolayers and $7 \times 7 \text{ mm}^2$ porous 3D cardiac tissue patches (Fig. 1AB, Movie 1). Elliptical pores in the patches facilitated nutrient transport and enabled the formation of uniformly dense and aligned cardiac tissue [26] (Fig. 1C and S3). Immunostaining for cardiac markers revealed aligned cardiomyocytes that exhibited cross-striated patterns of troponin T (cTnT, Fig. 1D), myosin heavy chain (MHC, Fig. 1E) and α -actinin (SAA, Fig. 2A), indicative of well-developed sarcomeric structures. Additionally, N-cadherin and connexin-43 (Cx43) immunostaining demonstrated presence of intercellular gap and adherens junctions, respectively, suggestive of functional electromechanical coupling between cardiomyocytes (Fig. 1F and 2A). Confocal analysis of non-cardiac cell markers revealed the presence of SM22 α^+ smooth muscle cells (Fig. 1F) that resided primarily at the patch surface along with vWF⁺/Vim⁺ fibroblasts (Fig. S4), while vWF⁺/Vim⁺ endothelial cells (Fig. 1F) occupied the patch interior (Fig. S4). Similar to 3D patches, 2D monolayer cultures also contained cross-striated hESC-CMs (Fig. 2B) interspersed with both fibroblasts and vascular cells (data not shown). Importantly, hESC-CMs in 3D tissue patches exhibited significantly longer sarcomeres than in 2D monolayers (2.09 ± 0.02 vs. 1.77 ± 0.01 μm , Fig. 2C,D), reaching optimal sarcomere length measured in adult human cardiomyocytes [31].

Time course of cardiac gene expression

Using qRT-PCR, we assessed expression of pluripotency and cardiac-specific genes (for the list of primers see Table S1) in undifferentiated hESCs, differentiated hESCs, and cardiac patches and monolayers at different times of culture. As expected, OCT4 and NANOG expression decreased significantly during hESC differentiation and virtually disappeared in patches (Fig. 3A,B) and monolayers (not shown). Early cardiac markers ISL1 and GATA4 increased with cardiac differentiation, with ISL1 decreasing during subsequent patch culture (Fig. 3C). Consistent with human ventricular development [32, 33], gene expression levels of β MHC and MLC2v increased and α MHC decreased during patch culture, yielding a significant increase in β MHC/ α MHC and MLC2v/MLC2a expression ratios (Fig. 3D-F). Furthermore, expression of genes that determine AP shape and propagation (Fig. 3G) either increased with tissue patch culture (Cx43, Na_v1.5, K_v4.3) or remained unchanged (Kir2.1, Ca_v1.2). Interestingly, genes crucial for excitation-contraction (E-C) coupling (CASQ2, SERCA2) were significantly upregulated during 2-week patch culture (Fig. 3H,I), showing a 170-fold increase in calsequestrin expression that was previously shown to be lacking in 2D cultured hESC-CMs [13, 14]. Compared to 2D monolayers, hESC-CMs in 3D patches had significantly upregulated genes required for cardiac E-C coupling (Fig. 3H,I) and contractile function (cTnT, α MHC, Fig. S5). In contrast, culture environment (2D vs. 3D) had no major influence on genes important for cardiac electrical function (Fig. 3G).

Action potential conduction in cardiac patches and monolayers

Optical mapping of transmembrane potentials (Fig. 4A) revealed macroscopically continuous action potential propagation in both tissue patches (Fig. 4B, Movie 2) and monolayers (Fig. 4C, Movie 3). Interestingly, conduction velocity (CV) in cardiac patches increased linearly with hESC-CM purity, reaching 25.1 cm/s in patches made of 90% cardiomyocytes (Fig. 4D). Compared to age- and purity- (48-65%) matched 2D monolayers, 3D patches exhibited significantly higher CVs (9.76 ± 1.0 vs. 5.17 ± 0.7 cm/s, Fig. 4D), but not significantly different action potential durations (APD, 338 ± 30 vs. 278 ± 20 ms, Fig. 4E) or maximum capture rates (MCR, 2.6 ± 0.2 vs. 2.6 ± 0.1 Hz, Fig. 4F). In contrast to CVs, neither APDs nor MCRs in patches or monolayers showed significant correlation with hESC-CM purity. Both CV and APD in patches decreased with increased pacing frequency (electrical restitution), while monolayers showed only significant APD restitution (Fig. S6A,B). Thus, 3D cardiac patches appeared to form a more functional electrical syncytium than 2D monolayers.

Contractile properties of cardiac tissue patches

Assessment of isometric force generation revealed that electrically stimulated cardiac tissue patches generated active forces (twitch contractions) that increased with applied stretch in a Frank-Starling-like fashion (Fig. 5A,B). Maximum active (contractile) forces averaged to 3.0 ± 1.1 mN and showed no significant correlation with hESC-CM purity (Fig. 5C). Based on measurements of the patch width (7 mm), thickness (73.6 ± 6.6 μ m, Fig. S3), percent pore area in the patch (porosity, $39.5 \pm 4.9\%$), and distribution of cell alignments [30], we estimated the average contractile stress in our cardiac patches to be 11.8 ± 4.5 mN/mm². Since methods to calculate contractile stress amplitudes may vary among different research groups, we also calculated a functional parameter that can serve to quantify the “efficiency of a cardiac tissue engineering system” by dividing the contractile force amplitude in each cardiac patch (system output) with the number of cardiomyocytes used to make the patch (system input). The calculated contractile-force-per-input-cardiomyocyte in our patches averaged to 5.7 ± 1.0 nN/cell and decreased with increase in hESC-CM purity (Fig. 5D). Similar to active forces, passive tension in patches increased with applied stretch (Fig. 5B), but significantly decreased with hESC-CM purity (Fig. S7A), yielding a linear increase in the active:passive force ratio for patches with higher hESC-CM purity (Fig. S7B). Along

with being mechanically softer, patches made with higher percent cardiomyocytes also displayed shorter twitch rise times (Fig. S7C,D).

Finally, we tested the sensitivity of engineered cardiac tissue patches to beta-adrenergic stimulation. Increasing doses of β -adrenergic agonist isoproterenol caused an expected increase in spontaneous contraction frequency (data not shown) as well as a sigmoidal increase in twitch amplitude (up to $\sim 1.7 \pm 0.3$ fold with EC_{50} of 95.1 nM, Fig. 6A,B). Furthermore, increasing isoproterenol concentrations yielded significant shortening of twitch rise time (Fig. 6C) indicative of accelerated force generation. Together with the results of structural, gene expression, and electrophysiological analyses, the high contractile stresses and inotropic responsiveness to adrenergic stimulation collectively demonstrated advanced functional maturation of hESC-CMs in the cardiac tissue patches.

Discussion

In this study, we generated relatively large, aligned, and highly functional cardiac tissue patches using human embryonic stem cell-derived cardiomyocytes. Our results demonstrate that after only two weeks of culture, the optimized 3D environment of a cardiac tissue patch can yield advanced levels of structural and functional maturation of hESC-CMs, as evidenced by their mature sarcomeric organization, enhanced cardiac gene expression profiles, and robust functional output. Specifically, we show that: 1) aligned human heart tissues can be engineered with a sheet-like (rather than cable) geometry and exhibit both high CVs (up to 25.1 cm/s) and contractile forces and stresses (3.0 mN and 11.8 mN/mm²), 2) initial hESC-CM purity uniquely determines the electromechanical properties of the engineered myocardium, and 3) hESC-CM maturation in optimized 3D patch environment is enhanced relative to that in standard 2D cultures. Additionally, engineered cardiac tissue patches demonstrated significant adrenergic responsiveness, confirming the advanced maturation state of hESC-CMs. These results build on our previous studies that established the optimal *in vitro* conditions for the formation of mouse ESC-derived myocardium and demonstrated a requisite role of non-cardiomyocytes in this process [26]. Similarly, in this study, the formation and/or maturation of functional human myocardium may have been supported by the presence of endothelial, smooth muscle, and fibroblastic cells in the patch. These cells have been differentiated together with cardiomyocytes from the same hESC source, thus avoiding the need for supplementation with exogenous non-cardiac cells, as employed in previous studies [21, 23, 34].

Methods for engineering contractile human cardiac tissues from pluripotent stem cell-derived cardiomyocytes have involved the use of scaffold-free cell sheets [35], porous polymer scaffolds [34], and different hydrogels, including collagen [21-23, 36], fibrin [19], and cardiac ECM-derived hydrogels [37]. Among these studies, only four [19, 21-23] have assessed tissue contractile function and only one [21] has also assessed electrical function of the engineered myocardium. Compared to these reports, cardiac tissue patches generated in our study exhibit 2.2-180-fold and 2.6-150-fold higher contractile force and stress generation, respectively (see Table S2 for all previous reports on functional assessment of primary and stem cell-derived engineered cardiac tissues). Due to a lack of standardized methods to calculate contractile stress amplitude (e.g. in geometrically complex tissues), we also introduced a unifying metric for quantifying the efficiency of a cardiac tissue engineering approach by dividing the maximum contractile force of a cardiac construct by the number of cardiomyocytes used for its production. Using this metric, we further showed 4-700-fold higher efficiency of our cardiac tissue approach (average force-per-CM ratio of 5.7 nN/cell) than previously achieved (Fig. 5D, Table S2). In addition, the maximum velocities of action potential propagation achieved in this study (at 80-90% hESC-CM purity) represent a 5-fold increase compared to previous reports in human engineered tissues

[21] (Table S2). Overall, cardiac tissue patches exhibited high contractile stresses and conduction velocities that are only 2-3 times lower than those reported for adult human myocardium (Table S2).

We also explored the effects of the initial hESC-CM purity on electromechanical properties of the engineered cardiac tissues. We found that higher CM purities produced faster CVs but had no significant effect on contractile force production (at least for 48-90% purity), thus yielding a decrease in force-per-input-cardiomyocyte ratio. While higher CM purity (i.e. less non-myocytes) would expectedly facilitate cardiomyocyte contacts and increase CV, lack of force augmentation may result from a limit on the CM survival in a given patch volume. Thus, the use of hydrogels seeded at low density with high purity of hESC-CMs may be the most efficient strategy to maximize CM survival and electromechanical output of 3D engineered myocardium. This strategy could be used for the optimal design of high-throughput screening platforms that employ 3D functional engineered cardiac tissues while utilizing minimum numbers of human CMs.

Furthermore, we demonstrated that relative to standard 2D culture, 3D patch environment significantly enhanced hESC-CM maturation. Specifically, on a structural level, 3D culture promoted an increase in sarcomere length to values comparable to those of adult human cardiomyocytes. Previously, sarcomere lengthening in hiPSC- and hESC-CMs was induced through mechanical stretch [21]; however, achieved sarcomere lengths remained at sub-adult values [31]. Interestingly, the 3D patch culture also enhanced expression of genes determining cardiac contractile function and Ca^{2+} handling (cTnT, α MHC, SERCA2, CASQ2) but not electrical function (Cx43, $\text{Na}_v1.5$, Kir2.1, $\text{Ca}_v1.2$). Combined with studies showing high CVs in relatively pure hiPSC-CM monolayers [20], our results suggest that 2D monolayers and 3D sheet-like patches may be of similar utility for studies of cardiac impulse propagation and related arrhythmogenesis. On the other hand, the advanced structural and contractile characteristics of hESC-CMs in 3D cardiac patches would render them preferred over 2D monolayers for physiological and pharmacological studies of cardiac contractile function and excitation-contraction coupling.

In support of this notion, cardiac patches were found to exhibit a robust inotropic response (i.e. an increased amplitude and faster kinetics of force generation) to a β -adrenergic agonist, isoproterenol. The inotropic effects of isoproterenol are characteristic of the adult human myocardium and require a functional sarcoplasmic reticulum, capable of releasing larger amounts of Ca^{2+} more quickly upon adrenergic stimulation [38, 39]. While this effect has been previously observed in some hESC-CM preparations [21, 40] but not others [18, 19], the relative sensitivity of the inotropic response to isoproterenol was not assessed. Our measured EC_{50} of 95.1 nM falls in the 30-160 nM range reported for adult human ventricular tissue [41, 42], suggesting a near-physiological sensitivity to adrenergic stimulation.

Conclusion

We have established methodologies for successful engineering of a highly functional human cardiac tissue patch starting from hESC-derived cardiomyocytes. The structural and functional properties of these 3D tissue patches, along with the resultant maturation state of hESC-CMs, surpass all previous reports for engineered human myocardium and currently provide the closest *in vitro* approximation of native human heart tissue. Overall, the use of non-genetic methods to purify human pluripotent stem cell-derived CMs, the derivation of vascular cells along with cardiomyocytes, aligned tissue structure, and robust functional output of the obtained cardiac patches all warrant the future use of this tissue-engineering

platform for predictive drug and toxicology testing and development of effective cell-based cardiac therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structural properties of human cardiac tissue patches

A) Representative 2-week old cardiac tissue patch anchored within a Velcro frame. B-C) Staggered elliptical pores within the patch (B) are surrounded by densely packed and aligned cells (C). D) hESC-CMs in 2-week old cardiac tissue patches are aligned and show cross-striated patterning of cardiac Troponin T (cTnT). Fibroblasts positive for vimentin (Vim) are interspersed among hESC-CMs. E) hESC-CMs also exhibit cross-striated pattern of myosin heavy chain (MHC) and sarcomeric α -actinin (SAA). F) Cardiac patches show evidence of mechanical coupling (N-cadherin) between hESC-CMs as well as presence of smooth muscle cells (SM22 α). Data shown for patches made with 70% hESC-CMs.

Figure 2. hESC-CMs in 3D patches exhibit longer sarcomeres than in 2D monolayers

A) Representative immunostainings of hESC-CMs in 2-week old tissue patch coupled by connexin-43 gap junctions. B) hESC-CMs in 2-week old monolayers. C) Histogram distribution of sarcomere lengths in 2-week old patches and monolayers made with 48-65% hESC-CMs. Note increased sarcomere length in patches vs. monolayers. D) Median and quartile sarcomere lengths in monolayers (n=58 hESC-CMs) and patches (n=106 hESC-CMs). *p<0.0001.

Figure 3. Quantitative gene expression during patch and monolayer culture

A-C) qPCR readout of pluripotency (*OCT4* and *NANOG*) and cardiac progenitor (*ISL1* and *GATA4*) genes in undifferentiated hESCs (ES), beating cell clusters (PM0), and cardiac patches at 4 days (P4D), 1 week (P1W), and 2 weeks (P2W) of culture. D-F) qPCR readout of contractile function genes: MHC, myosin heavy chain; cTnT, cardiac Troponin T; *MLC2v/a*, Myosin light chain 2 ventricular/atrial. G-I) qPCR readout of electrical function (*Nav1.5*, fast Na⁺ channel; *Kir2.1*, inward rectifier K⁺ channel; *Kv4.3*, transient outward K⁺ channel; *Cav1.2*, L-type Ca²⁺ channel) and excitation-contraction (*SERCA2*, SR Ca²⁺-ATPase; *CASQ2*, calsequestrin) genes in patches and monolayers of the same age (M1W and M2W, 1 and 2 week monolayer) and hESC-CM purity (60-65%). All data except F are shown relative to PM0 group. n = 2–4 biological × 3 technical replicates. p < 0.05 from: *, PM0; **, all other groups; †, ES; #, another group.

Figure 4. Action potential propagation in cardiac patches and monolayers

A) Representative optically mapped action potential traces in a 2-week old cardiac patch at various pacing rates. B,C) Representative isochrone maps during 1 Hz point stimulation of a 2-week old cardiac patch (B) and monolayer (C). D-F) Conduction velocity (D), action potential duration (E), and maximum capture rate (F) during point stimulation of cardiac patches and monolayers as a function of hESC-CM purity. $\dagger p < 0.05$ between patches and monolayers (48% < hESC-CM purity < 65%).

Figure 5. Contractile properties of cardiac tissue patches

A) Representative active (contractile) force traces during progressive stretch of an electrically stimulated (1Hz) 2-week cardiac patch. B) Corresponding active and passive force-length relationships in cardiac patches made with 57%-65% hESC-CMs (n=5). C) Maximum contractile force (at 1 Hz stimulation) in 2-week patches as a function of hESC-CM purity. D) Contractile force per input hESC-CM as a function of cardiomyocyte purity; $p < 0.03$ for linear trend.

Figure 6. Inotropic response of cardiac tissue patches to beta-adrenergic stimulation

A) Representative active force traces in a tissue patch made with 55% hESC-CMs during application of increasing doses of isoproterenol (measured at 10% stretch, 1 Hz stimulation, and 0.9 mM extracellular [Ca²⁺]). B-C) Contractile force amplitude (B) and twitch kinetics (C) as a function of isoproterenol concentration (n=4).