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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/mm^2, 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, 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.

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1. 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

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(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/mm^2) and conduction velocities (<4.9 cm/s), an order of magnitude lower than adult human myocardium (20–45 mN/mm^2 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.

2. Materials and methods

2.1. 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% O_2, 5% CO_2, 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% O_2, 5% CO_2, 37 °C) to ambient oxygen condition (5% CO_2, 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.

2.2. 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 h, followed by 0.25% trypsin/EDTA (Cellgro) with gentle shaking in a 37 °C water bath for 5–7 min. Single-cell suspension was achieved by triturating with equal volume of 50% FBS/DMEM + 20 μg/ml DNase (Calbiochem).

2.3. 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 (5×10^6 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.

2.4. Cardiac patch fabrication and culture

To generate aligned 3D human cardiac tissue patches, 7 × 7 mm^2 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 2× cardiac media) was mixed with 1×10^6 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 1 h to polymerize. Resulting cardiac patches were cultured with rocking in cardiac medium (5% FBS/DMEM with 1 mM sodium pyruvate (Gibco), 2 mM Glutamine (Gibco), 0.1 mM non-essential amino acids (Gibco), 50 μg/ml Ascorbic Acid (Sigma), and 0.45 mM monothioglycerol (Sigma)) for 4–14 days. Culture media was supplemented daily with 1 mg/ml aminocaproic acid (Sigma) to prevent fibrin degradation. 10 μM 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.

2.5. 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 (4× 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].

2.6. 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 0.9 mM Ca^{2+} Tyrode's solution during 1 Hz electrical stimulation at 10% stretch.

2.7. Statistical analysis

Experimental data reported as mean ± 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.

Additional details and assessment methodologies are provided in Supplemental Materials.

3. Results

3.1. 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.

3.2. 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 × 7 mm^2 porous 3D cardiac tissue patches (Fig. 1A–B, Movie 1). Elliptical pores in the patches facilitated nutrient transport and

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