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Dynamic culture yields engineered myocardium with near-adult functional output

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ABSTRACT

Engineered cardiac tissues hold promise for cell therapy and drug development, but exhibit inadequate function and maturity. In this study, we sought to significantly improve the function and maturation of rat and human engineered cardiac tissues. We developed dynamic, free-floating culture conditions for engineering "cardiobundles", 3-dimensional cylindrical tissues made from neonatal rat cardiomyocytes or human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) embedded in fibrin-based hydrogel. Compared to static culture, 2-week dynamic culture of neonatal rat cardiobundles significantly increased expression of sarcomeric proteins, cardiomyocyte size (~2.1-fold), contractile force (~3.5-fold), and conduction velocity of action potentials (~1.4-fold). The average contractile force per cross-sectional area (59.7 mN/mm^2) and conduction velocity (52.5 cm/s) matched or approached those of adult rat myocardium, respectively. The inferior function of statically cultured cardiobundles was rescued by transfer to dynamic conditions, which was accompanied by an increase in mTORC1 activity and decline in AMPK phosphorylation and was blocked by rapamycin. Furthermore, dynamic culture effects did not stimulate ERK1/2 pathway and were insensitive to blockers of mechanosensitive channels, suggesting increased nutrient availability rather than mechanical stimulation as the upstream activator of mTORC1. Direct comparison with phenylephrine treatment confirmed that dynamic culture promoted physiological cardiomyocyte growth rather than pathological hypertrophy. Optimized dynamic culture conditions also augmented function of human cardiobundles made reproducibly from cardiomyocytes derived from multiple hPSC lines, resulting in significantly increased contraction force (~2.5-fold) and conduction velocity (~1.4-fold). The average specific force of 23.2 mN/mm^2 and conduction velocity of 25.8 cm/s approached the functional metrics of adult human myocardium. In conclusion, we have developed a versatile methodology for engineering cardiac tissues with a near-adult functional output without the need for exogenous electrical or mechanical stimulation, and have identified mTOR signaling as an important mechanism for advancing tissue maturation and function *in vitro*.

1. Introduction

Engineered cardiac tissues can be used for *in vitro* studies of heart development [1,2], modeling of cardiovascular disease [3–5], and high-throughput screening of drug effects on cardiomyocyte (CM) survival and function [6,7]. However, fidelity of these assays is currently limited by poor functional properties of cultured CMs compared to adult CMs [8,9]. Specifically, engineered 3D cardiac tissue constructs generate contractile stresses that are an order of magnitude lower than those of adult ventricular myocardium and conduct action potentials at a significantly slower speed, likely due to CM immaturity and a low volume fraction of CMs within the tissue [10]. Engineering tissues that accurately mimic the structure and function of the native myocardium would enable more predictive *in vitro* studies and promote cell therapies for myocardial infarction and heart failure [2,11]. In the latter case, achieving highest velocity of action potential conduction and contractile stress would be two fundamental requirements to ensure electrical safety and functional efficacy of the therapy, respectively.

The adult heart consists of densely packed myocytes with high metabolic demand, which necessitates at least one capillary per CM to supply nutrients [12]. Local depletion of oxygen has been observed in the vicinity of cultured CMs [13–15], highlighting the

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need to enhance nutrient and oxygen transport in 3D engineered cardiac tissues. Previous studies have utilized tissue perfusion [15–17] or bioreactor culture [18–20] to improve mass transport in engineered myocardium, with low-shear conditions being preferable due to CM susceptibility to shear-induced damage [17,20,21]. Many of these pioneering reports involved the use of porous polymer scaffolds; however, natural hydrogels (fibrin, collagen) have proven superior for the generation of highly functional cardiac tissues [22–24] because they support uniform cell density, macroscopic contractions, and application of tension to support myocyte spreading and alignment [25,26].

We therefore sought to develop a versatile approach to culture hydrogel-based cardiac tissue constructs under low-shear dynamic conditions that would support high tissue cellularity while maintaining mechanical tension requisite for cell survival and functional cardiogenesis. Specifically, we designed cylindrically shaped engineered cardiac tissues ("cardiobundles") anchored within porous flexible frames that supported both a chronic auxotonic loading and free-floating culture of CM tissues. Dynamic culture was applied using standardized tissue culture plates and platform rockers, eliminating the need for custom-designed bioreactors or perfusion chambers. The effects of dynamic culture on tissue density, CM size, and electrical and mechanical function were first established for cardiobundles made of neonatal rat CMs, followed by the application of optimized conditions to hPSC-CMs. For both rat and human tissues, we observed significant improvements in myocardial mass, CM maturation, and tissue function with dynamic culture, and identified mTOR signaling as a critical mechanistic component of this response. The resulting cardiac tissue model is simple for implementation and represents promising platform for future applications in cardiac disease modeling, drug screening, and regenerative therapy.

2. Methods

Additional details and assessment methodologies are provided in Supplemental Materials.

2.1. Neonatal rat CM isolation and fabrication of engineered cardiobundles

Neonatal rat ventricular myocytes (NRVMs) were isolated from the hearts of 2-day-old Sprague-Dawley rats using previously described methods [19,27] and embedded in a fibrin-based hydrogel cast into 7 mm × 2 mm troughs of pre-fabricated polydimethylsiloxane (PDMS) molds (Fig. S1). Obtained cardiobundles, each constructed from 3.75×10^5 cells, were anchored at each end by a porous nylon frame (Cerex®) and removed from troughs to allow free movement in culture media. Cardiobundles were cultured on a static (non-moving) or dynamic (rocking, ± 30° tilt, 0.4 Hz [28], Supplemental Video 1) platform for a period of 14 days. For some experiments, tissues were switched between static and dynamic conditions on day 7.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2016.09.024.

2.2. hPSC-CM differentiation and cardiobundle fabrication

Human cardiobundles were constructed using CMs differentiated from 2 hESC lines (H9, RUES2) and one iPSC line (derived at the Duke University Core Facility). Cardiac differentiation was carried out using small molecules CHIR99021 and IWP-2 to upregulate and inhibit Wnt signaling, respectively, as previously described [29]. In some studies, hPSC-CMs were purified using glucose-free chemically defined medium (CDM3) applied at differentiation days 10–12 [30,31]. After 12–22 days of differentiation, derived hPSC-CMs were utilized to fabricate cardiobundles as described for NRVMs.

2.3. Immunohistology

Intact cardiobundles and 10 μm thick cross-sections were fixed and immunostained using standard techniques.

2.4. Western blotting and qPCR

Expression of cardiac genes and proteins was analyzed using standard qPCR protocols and Western blot.

2.5. Optical mapping of action potential propagation

Optical mapping of action potentials was performed as previously described [32,33]. Cardiobundles were stained with a transmembrane voltage-sensitive dye (di-4-ANEPPS) and paced at different rates by suprathreshold point stimulus to map propagation of action potentials. Calcium transients were imaged using lentiviral expression of a genetically encoded calcium indicator (GCaMP6) driven by a myocyte-specific promoter (MHCK7) [34], or using Rhod-2 AM.

2.6. Measurement of isometric contractile force generation

Isometric contractile forces were measured as previously described [33,35]. Cardiobundles were immersed in Tyrode's solution containing 1.8 mM $CaCl_2$ and connected to a force transducer. Contractions were elicited by electric field stimulus from parallel platinum electrodes.

2.7. Statistics

Data are expressed as mean ± SEM. Statistically significant differences were determined by one-way ANOVA with Tukey's *post hoc* test and $p < 0.05$ was considered significantly different.

3. Results

3.1. Dynamic culture of cardiobundles stimulates growth of neonatal rat cardiomyocytes

After 2 weeks of culture, cardiobundles consisted of uniformly aligned, cross-striated cardiomyocytes that robustly expressed electrical and mechanical junctions (Fig. 1A, Fig. S2). In both static and dynamic conditions, NRVM cardiobundles contracted synchronously and spontaneously at rates that initially increased and then decreased to completely cease after culture day 10 (Fig. S3, Supplemental Video 2). In longitudinal sections, CMs cultured under dynamic conditions appeared larger and more densely packed than CMs in static culture (Fig. 1A). In transverse cross-sections (Fig. 1B), dynamically cultured cardiobundles exhibited a 2.5-fold larger muscle area (Fig. 1C, right), as quantified from F-actin stainings (Fig. S4), and only 1.2-fold higher nuclei number (Fig. 1D–E) compared to statically cultured cardiobundles. Similar cellularities in static and dynamic culture were confirmed by two additional assays (Fig. S5). Consistent with comparable cell numbers, static and dynamic cardiobundles had comparable expressions of hypoxia marker PHD2 (Fig. S6), while PHD3 expression was 2-fold higher in static condition (Fig. S6). Regarding previous reports of ~5-fold increased PHD2 and ~40-fold increased PHD3 expression in 0.5–1% oxygen vs. normoxia [36,37], these results suggested that static culture exhibited only a mild hypoxia relative to dynamic culture. From transverse cross-sections, we estimated a