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Physiologic force-frequency response in engineered heart muscle by electromechanical stimulation

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ABSTRACT

A hallmark of mature mammalian ventricular myocardium is a positive force-frequency relationship (FFR). Despite evidence of organotypic structural and molecular maturation, a positive FFR has not been observed in mammalian tissue engineered heart muscle. We hypothesized that concurrent mechanical and electrical stimulation at frequencies matching physiological heart rate will result in functional maturation. We investigated the role of biomimetic mechanical and electrical stimulation in functional maturation in engineered heart muscle (EHM). Following tissue consolidation, EHM were subjected to electrical field stimulation at 0, 2, 4, or 6 Hz for 5 days, while strained on flexible poles to facilitate auxotonic contractions. EHM stimulated at 2 and 4 Hz displayed a similarly enhanced inotropic reserve, but a clearly diverging FFR. The positive FFR in 4 Hz stimulated EHM was associated with reduced calcium sensitivity, frequency-dependent acceleration of relaxation, and enhanced post-rest potentiation. This was paralleled on the cellular level with improved calcium storage and release capacity of the sarcoplasmic reticulum and enhanced T-tubulation. We conclude that electro-mechanical stimulation at a physiological frequency supports functional maturation in mammalian EHM. The observed positive FFR in EHM has important implications for the applicability of EHM in cardiovascular research.

1. Introduction

Mature mammalian ventricular myocardium exhibits a positive force-preload relationship (Frank-Starling mechanism) and a positive force–frequency relationship (FFR; Bowditch phenomenon), both of which are of critical importance for the contractile performance of healthy myocardium [1]. Loss of both mechanisms along with fundamental alterations in excitation-contraction coupling are key features in heart failure [2,3]. The positive FFR seems to be intrinsically dependent on the maturity of the intracellular calcium stores, the sarcoplasmic reticulum (SR), and T-tubulation, which develop postnatally, allowing for rapid intracellular recycling of calcium during systole and diastole [4–6].

Tissue engineering aims at recapitulating myocardial physiology and pathology *in vitro* [7]. Over the past two decades, morphological (e.g., tissue anisotropy), molecular (e.g., low expression of fetal genes), and functional data (e.g., positive Frank-Starling mechanism) have provided compelling evidence in support of the notion that tissue engineered myocardium is more mature than classical two-dimensional cultures [8,9]. Mechanical [10,11] and electrical [12,13] stimulation were associated with enhanced

Abbreviations: AAV, adenovirus-associated virus; CSQ2, Calsequestrin 2; DMS, dynamic mechanical stimulation; EHM, Engineered Heart Muscle; FDAR, frequency dependent acceleration of relaxation; FFR, force-frequency relationship; FOC, force of contraction; LTCC, L-type Calcium channel; PBS, phosphate buffered solution; PRP, post-rest potentiation; RyR2, Ryanodine receptor 2; SR, Sarcoplasmic Reticulum; SERCA2a, sarco/endoplasmic reticulum; Ca^{2+}, ATPase; WGA, wheat germ agglutinin.

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maturation. Dynamic mechanical stimulation (DMS) designed to facilitate auxotonic contractions against a defined resilient load was most effective in enhancing maturation in engineered heart muscle (EHM) [14,15]. Similarly, optimization of electrical field stimulation improved contractile parameters [12,13,16,17] and enhanced hypertrophic growth [18]. Although intuitively of great interest, experiments combining these paradigms in a physiology-adapted manner have not been reported. Similarly, no studies to date have enabled the generation of tissue engineered myocardium with a mature, i.e., positive FFR, which is a classical hallmark of mammalian ventricular myocardium [1,8]. Importantly, the positive FFR is critical for the *in vitro* screening and validation of chronotropic and inotropic drugs.

Because heart muscle development and maturation depend on electro-mechanical inputs, we reasoned that supporting auxotonically contracting, i.e., mechanically loaded, EHM by electrical stimulation at frequencies observed in neonatal heart (~4–8 Hz in 1–14 day old rats [19]) would enhance functional maturation and recapitulate postnatal development with SR-functionality and T-tubulation. Using this approach, we achieved for the first time a positive FFR in mammalian tissue engineered myocardium and concomitantly identified maturation of the sarcoplasmic calcium handling machinery as underlying mechanism. These results provide strong arguments for the use of electro-mechanical stimulation regimens as a means to achieve a fundamental physiologic property necessary for the utilization of EHM for *in vitro* studies of cardiovascular physiology and pharmacology.

2. Results

2.1. Electro-mechanical stimulation matures contractile function of EHM

EHMs were cultured under dynamic mechanical stimulation (DMS; Fig. 1) applied after a consolidation phase to support auxotonic contractions [14]. On culture day 8, spontaneous beating frequency of EHM was 1.23 ± 0.15 Hz (n = 50). Within 48 h, EHMs could be reproducibly captured at 2 and 4 Hz, but not at 6 Hz. After 5 days of electro-mechanical stimulation (culture day 13), we observed a marked drop in spontaneous beating frequency – an indicator of maturation, that was most pronounced in the 4 Hz group (Unstimulated: 0.63 ± 0.11 Hz [n = 17]; 2 Hz group: 0.45 ± 0.15 Hz [n = 16]; 4 Hz group: 0.35 ± 0.15 Hz [n = 17]). Electrical stimulation increased force of contraction (FOC) of EHMs at maximum extracellular calcium (2.8 mmol/L) compared to mechanically stimulated EHM (Unstimulated: 0.65 ± 0.08 mN [n = 16]; 2 Hz: 0.95 ± 0.11 mN [n = 11]; 4 Hz: 1.2 ± 0.08 mN [n = 16]; Fig. 2A). Notably, a significant shift in the calcium sensitivity was observed in the 4 Hz group (EC_{50} for calcium: 0.45 ± 0.02 mmol/L [Unstim]; 0.45 ± 0.03 mmol/L [2 Hz]; 0.74 ± 0.06 mmol/L [4 Hz]), suggesting maturation of the calcium handling machinery. Importantly, electrical stimulation did not influence total cell number (combined average from 22 EHMs: 6.1 ± 0.3 × 10^5; Figure S1A), cell viability after enzymatic dispersion of EHM (combined average from 22 EHMs: 45 ± 2%; Figure S1B), cardiomyocyte number (combined average from 22 EHMs: 0.75 ± 0.07 × 10^5; Figure S1C), and cardiomyocyte size assessed by cytometry for actinin fluorescence signal intensity per cell (average from 8 to 10 EHM per group; Figure S1D). The latter data were confirmed by morphometric quantification of the cardiomyocyte area (Unstimulated: 548 ± 62 μm^2 [n = 11 cardiomyocytes]; 2 Hz: 564 ± 46 μm^2 [n = 9 cardiomyocytes]; 4 Hz: 560 ± 38 μm^2 [n = 17 cardiomyocytes]; Figure S1D). Note that cardiomyocytes from postnatal day-13 were larger (931 ± 61 μm^2; n = 20 cardiomyocytes) in comparison to any of the investigated EHM groups ($P < 0.05$ by ANOVA with Bonferroni multiple comparison test).

2.2. Positive force-frequency relationship in 4 Hz stimulated EHM

A classical property of mature non-failing ventricular myocardium is a positive FFR, associated typically with frequency-dependent acceleration of relaxation (FDAR) [1]. Neither a positive FFR nor FDAR have been reported in any mammalian model of tissue engineered myocardium [8]. In EHM developed under electro-mechanical stimulation at 4 Hz – a physiological heart rate in 1 day old rats [19,20] - we now find for the first time a clearly positive FFR (Fig. 2B–C), demonstrating that electrical stimulation at physiological frequency is critical for functional maturation. Consistently, we observed in the 4 Hz EHM a markedly shortened relaxation time and enhanced FDAR (Fig. 2D).

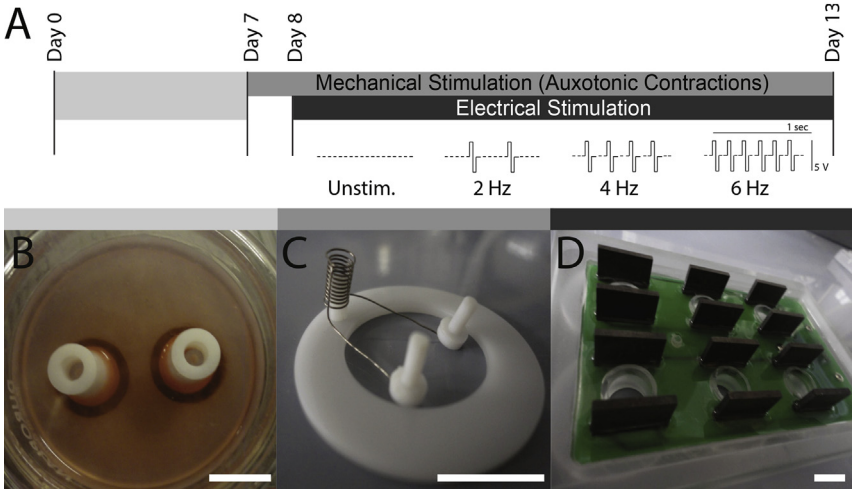

Fig. 1. Experimental design. A. EHMs were cultured over a 13-day period, with an 8-day consolidation phase (7 days in casting mold [B] plus 1 day on DMS [C]) followed by a 5 day maturation phase under electro-mechanical stimulation (DMS plus 3 ms biphasic 5 V pulses at 2, 4, or 6 Hz); unstimulated, i.e. spontaneously and auxotonically beating EHMs (Unstim.) served as controls. B. Casting mold with two already condensed EHMs. C. Custom made stretch device for DMS to support auxotonic contractions of EHMs. D. C-dish stimulation plate (Ionoptix) used to deliver square biphasic pulses to EHMs via graphite electrodes. Scale bars: 10 mm.