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Effect of engineered anisotropy on the susceptibility of human pluripotent stem cell-derived ventricular cardiomyocytes to arrhythmias

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ABSTRACT

Human (h) pluripotent stem cells (PSC) such as embryonic stem cells (ESC) can be directed into cardiomyocytes (CMs), representing a potential unlimited cell source for disease modeling, cardiotoxicity screening and myocardial repair. Although the electrophysiology of single hESC-CMs is now better defined, their multi-cellular arrhythmogenicity has not been thoroughly assessed due to the lack of a suitable experimental platform. Indeed, the generation of ventricular (V) fibrillation requires single-cell triggers as well as sustained multi-cellular reentrant events. Although native VCMs are aligned in a highly organized fashion such that electrical conduction is anisotropic for coordinated contractions, hESC-derived CM (hESC-CM) clusters are heterogenous and randomly organized, and therefore not representative of native conditions. Here, we reported that engineered alignment of hESC-VCMs on biomimetic grooves uniquely led to physiologically relevant responses. Aligned but not isotropic control preparations showed distinct longitudinal (L) and transverse (T) conduction velocities (CV), resembling the native human V anisotropic ratio (AR = LCV/TCV = 1.8–2.0). Importantly, the total incidence of spontaneous and inducible arrhythmias significantly reduced from 57% in controls to 17–23% of aligned preparations, thereby providing a physiological baseline for assessing arrhythmogenicity. As such, promotion of pro-arrhythmic effect (e.g., spatial dispersion by β adrenergic stimulation) could be better predicted. Mechanistically, such anisotropy-induced electrical stability was not due to maturation of the cellular properties of hESC-VCMs but their physical arrangement. In conclusion, not only do functional anisotropic hESC-VCMs engineered by multi-scale topography represent a more accurate model for efficacious drug discovery and development as well as arrhythmogenicity screening (of pharmacological and genetic factors), but our approach may also lead to future transplantable prototypes with improved efficacy and safety against arrhythmias.

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1. Introduction

Ventricular fibrillation (VF) is the most common cause for sudden death in adults; heart failure patients are particularly prone to VF [1]. The generation of VF requires both a cellular trigger (e.g., action potential prolongation, early and delayed after depolarizations) as well as multi-cellular reentrant events (e.g., spiral wave reentry) [2]. Human (h) pluripotent stem cells (PSC) such as embryonic stem cells (ESC) can be directed into the cardiac lineage with high efficiency [3–5], presenting a potential unlimited source of cardiomyocytes (CMs) for disease modeling, cardiotoxicity screening and myocardial repair [6]. Although the electrophysiology of single hESC-CMs has been described in multiple studies

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[7–13], their multi-cellular arrhythmogenicity has not yet been systematically and thoroughly assessed due to the lack of a suitable experimental platform. Given that hESC-CMs are functionally immature at the single-cell level [9,14–16], such can serve as substrates for arrhythmias in multi-cellular preparations. In the native heart, ventricular (V) CMs are aligned in a highly organized manner such that the conduction of electrical signals is anisotropic with distinct transverse and longitudinal velocities for coordinated, directional electrical and contractile activities [17,18]. By contrast, hESC-CM clusters differentiated *in vitro* using either embryoid body (EB) formation [19] or even directed cardiac differentiation [5] are heterogenous, containing a mixed population of V, atrial and pacemaker derivatives, and *randomly* organized, and therefore not representative of native conditions [19,20]. Here we tested the hypothesis that physical alignment of hESC-VCMs by shrink-induced biomimetic multi-scale wrinkled substrates that mimic the physiological setting seen in the native heart would lead to functional anisotropy and electrophysiological stability against the formation of sustained arrhythmic events.

2. Materials and methods

2.1. HESC culture and ventricular specification

Electrophysiological heterogeneity is a known contributing factor to sustained reentry and arrhythmias. To avoid dispersion of refractoriness due to mixed chamber-specific subtypes from *in vitro* cardiac differentiation [21], we employed a ventricular specification protocol (Karakikes et al.; submitted for publication) modified from a hESC cardiogenic differentiation technique [22]. As gauged by patch-clamp action potential (AP) recordings (Fig. 1A), troponin T (TnT)-positive immunostaining (Fig. 1B), and dsRed expression under the transcriptional control of the ventricular-specific myosin light chain-2v (MLC2v) promoter (Fig. 1C,D), our revised protocol generates 89.4 ± 5.9% hESC-VCMs (Fig. 1E,F). In brief, undifferentiated hESCs (HES2 line, NIH code ES02) were cultured under feeder-free condition and passaged when the culture reached ~80% confluence. Stem cell culture medium (mTeSR1, Stem Cell Technologies, Vancouver, BC, Canada) was changed daily. For ventricular specification, hESC colonies were dissociated by dispase (1 mg/ml) into smaller clusters (50–100 cells) followed by culturing in differentiation media (StemPro34 50 μg/ml ascorbic acid and 2 mM GlutaMAX-I; Invitrogen, Carlsbad, CA) supplemented with cytokines and Wnt inhibitor as follows: day 1, BMP4 (1 ng/ml) and blebbistatin (5 μM); days 2–4.5, human recombinant BMP4 (10 ng/ml) and human recombinant Activin-A (5 ng/ml); days 4.5–7, IWR-1 (1 μM). Differentiation medium was replenished daily until day 18–20, at which time hESC-VCMs were dissociated and plated on control or aligned Matrigel coated polyethylene substrates for 7 days to allow establishment of intercellular electrical junctions before electrical recordings. Only intact preparations without structural or geometric defects were used for experiments.

2.2. Fabrication and characterization of wrinkle substrates

The fabrication method has been previously described [15]. Briefly, pre-stressed polyethylene shrink film was treated with oxygen plasma (Plasma Prep II, SPI Supplies) for 2, 5, 10, or 15 min to generate P2, P5, P10 and P15 substrates. The plasma treated film was then constrained on opposite sides, and was shrunk at 150 °C for 3 min to create micro-wrinkled masters, which were then replicated using soft lithography with PDMS to yield substrates for inducing cell alignment (Fig. 2A). Both scanning electron microscopy (SEM) and atomic force microscopy (AFM) were performed to characterize the wrinkles (Fig. 2B). For SEM, wrinkles were sputtering coated (Polaron SC7620) with 4 nm gold, and images were obtained at 1000× magnification (FEI Quanta 3D FEG). Wavelengths of wrinkles were obtained by analyzing the SEM images using an in-house MATLAB (MathWorks Inc., Natick, MA, USA) fast Fourier transform code. AFM was performed on a MFP-3D inverted optical microscope (Asylum Research, Santa Barbara, CA). The topographic of images were taken in tapping mode with a tip resonant frequency of about 75 kHz and force constant of 3 N/m. Data acquisition and analysis were performed with IGOR Pro 6.0 (Wavemetrics, Portland, OR).

2.3. Optical mapping

HESC-VCM preparations were loaded with 4 μM di-4-ANEPPS (Invitrogen, USA) for 20 min at room temperature in Tyrode's solution, consisting of (mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose, and 10 HEPES at pH 7.4, then washed twice before fluorescence imaging using a halogen light filtered by a 515 ± 35 nm band-pass excitation filter and a 590 nm high-pass emission filter. High-resolution optical mapping of AP and conduction properties was performed using a MiCam Ultima (SciMedia, CA, USA) with a 1× objective and a 1× condensing lens to yield a 10 × 10 mm^2 field of view. A custom heat plate was introduced to maintain the perfused Tyrode's solution at 37 °C. Data were collected at a sampling rate of 200 Hz and analyzed using BV_Ana software (SciMedia). Isoproterenol (Sigma–Aldrich, NY, USA) was applied by perfusion at the concentrations indicated.

2.4. Electrophysiology

Electrophysiological protocols were generated using a programmable stimulator (Master8, AMPI, Israel) with the stimuli delivered via a unipolar point-stimulation electrode (1.5× threshold, 10 ms duration) placed perpendicular to the preparation surface. Steady-state pacing was initiated at a rate of 0.5 Hz, except in the presence of isoproterenol when 1.5–2.0 Hz frequency was imposed to overdrive the positive chronotropic effect on intrinsic rhythm. The frequency was increased every minute in 0.1-Hz increments with a 5-sec pause. Mapping was performed during the

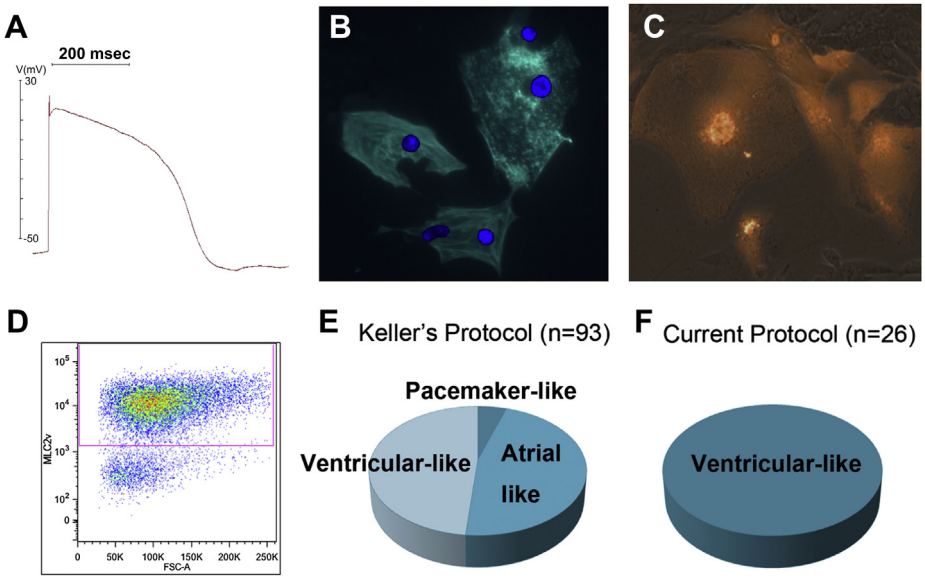

Fig. 1. *Ventricular specification of hESCs.* A) Representative AP supported a ventricular phenotype. B) Positive staining by cTNT and C) dsRed expression after transduction by LV-MLC2v-dsRed. D) Flow cytometry indicates that 89.4 ± 5.9% of the derivatives were MLC2v-dsRed-positive. A representative plot with a ~95% yield is shown. E and F) Pie charts showing the percentage distribution of ventricular-, atrial- and pacemaker-like AP phenotypes.