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Functional maturation of human pluripotent stem cell derived cardiomyocytes *in vitro* – Correlation between contraction force and electrophysiology

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ABSTRACT

Cardiomyocytes from human pluripotent stem cells (hPSC-CM) have many potential applications in disease modelling and drug target discovery but their phenotypic similarity to early fetal stages of cardiac development limits their applicability. In this study we compared contraction stresses of hPSC-CM to 2nd trimester human fetal derived cardiomyocytes (hFetal-CM) by imaging displacement of fluorescent beads by single contracting hPSC-CM, aligned by microcontact-printing on polyacrylamide gels. hPSC-CM showed distinctly lower contraction stress than cardiomyocytes isolated from hFetal-CM. To improve maturation of hPSC-CM *in vitro* we made use of commercial media optimized for cardiomyocyte maturation, which promoted significantly higher contraction stress in hPSC-compared with hFetal-CM. Accordingly, other features of cardiomyocyte maturation were observed, most strikingly increased upstroke velocities and action potential amplitudes, lower resting membrane potentials, improved sarcomeric organization and alterations in cardiac-specific gene expression. Performing contraction force and electrophysiology measurements on individual cardiomyocytes revealed strong correlations between an increase in contraction force and a rise of the upstroke velocity and action potential amplitude and with a decrease in the resting membrane potential.

We showed that under standard differentiation conditions hPSC-CM display lower contractile force than primary hFetal-CM and identified conditions under which a commercially available culture medium could induce molecular, morphological and functional maturation of hPSC-CM *in vitro*. These results are an important contribution for full implementation of hPSC-CM in cardiac disease modelling and drug discovery.

1. Introduction

The efficiency of cardiomyocyte differentiation of human pluripotent stem cells (hPSC) has greatly improved in recent years [1], with many reports describing more than 60% cardiomyocytes in differentiated cultures. For most laboratories, generating hPSC-derived cardiomyocytes (hPSC-CM) is no longer a major hurdle, providing opportunities for disease modelling, drug toxicity screening and identifying drug sensitivities of certain genotypes [2,3]. However, hPSC-CM are immature compared with human adult cardiomyocytes and express typical fetal cardiac genes [4], have immature electrophysiological properties such as low resting membrane potential and slow upstroke velocities [5], use glucose as major energy source as opposed to the fatty acids used by adult

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cardiomyocytes [6], have underdeveloped, (partially) disarrayed sarcomeres, heterogeneous shapes and are smaller in size [7,8]. Several studies have reported that the contraction stress of hPSC-CM using hydrogel-based technologies in two (2D) and three dimensions (3D) varied from 0.22 ± 0.70 mN/mm^2 to 11.8 ± 4.5 mN/mm^2 [9–12]. Furthermore, it has also been shown that contraction stress is dependent on substrate stiffness [13], emphasizing the necessity of using similar substrate stiffness for comparison of contraction stress values between different conditions.

Optimal implementation of hPSC-CM in human cardiac disease modelling and drug toxicity screening, would benefit from greater maturation of hPSC-CM. Therefore, it is important to understand which physiological parameters impact features of maturation. Candidate parameters that have been examined to date include long-term culture to mimic developmental gestation [14], substrate stiffness [15], cell patterning and alignment as occurs in the normal heart [16,17], electrical and mechanical stimulation [18], mechanical loading [19], interaction with other cell-types [20], and interference with signalling pathways thought to be of importance in heart development The outcomes of these studies have been variable: in assessing the effects of long-term culture, hPSC-CM were reported to withdraw from the cell-cycle and show ultrastructural maturation in 35 days [21] and increased conduction velocities in 2 months [22] but no improvements in contraction force were observed up to 2 or 3 months in culture [9,13] Following hPSC-CM for even longer, up to 4 months, both morphological and functional maturation were reported for both human embryonic stem cell (hESC) and human induced pluripotent stem cell (hiPS)-derived cardiomyocytes [14]. In assessing the role of substrate stiffness, neonatal rat ventricular myocytes (NRVM) cultured on a glass substrate (Young's Modulus ≈50 GPa), which is six orders of magnitude stiffer than heart tissue [23], showed decreased length to width ratios and increased stress fibre content compared to NRVM on substrates with Young's Modulus of 10 kPa [15,23]. Both stiffness and cell shape have regulatory roles in cytoskeletal architecture and extra cellular matrix (ECM) coupling [24]. In turn, the cytoskeletal architecture is known to regulate gene expression [25], impulse propagation [26], excitability [27] and contraction [16,28], demonstrating the regulatory role of cell shape on cardiac function. *In vivo,* adult ventricular cardiomyocytes are elongated and aligned along one axis with length/width ratios of 7:1 [17,24]. Accordingly, restraining cell shape to a more elongated phenotype reminiscent of that of adult cardiomyocytes improves cardiomyocyte structure and function [16,29]. Thyroid hormones also have an important influence on cardiac structure, electrophysiological functions and cardiac contractility in development *in vivo* [30], by directly regulating key structural and regulatory genes such as *α-MHC* and *β-MHC*, *SERCA2*, *PLN*, and various ion channels [31]. Moreover, triiodothyronine (T3) has been described as promoting electrophysiological and calcium handling maturation in mouse embryonic stem cell derived cardiomyocytes [32] and contraction maturation hiPS-CM [33].

Alterations in cardiomyocyte transcriptional, biochemical and functional cues, manifested by changes in electrophysiology, calcium handling, bioenergetics and contraction force, are inherent to progression from fetal to an adult state [8]. Consequently, *in vitro* cardiomyocyte maturation state can be assessed by analyses of these features. Nevertheless, the relationship and interdependence of these different parameters are poorly understood. Here, we found that hPSC-CM generate lower contraction stress than second trimester (16 week) human fetal cardiomyocytes (hFetal-CM) at the same substrate stiffness. Furthermore, by making use of different maturation media we were able to demonstrate that hPSC-CM displayed improved sarcomeric structure organization, stronger contraction stress, improved electrophysiological parameters and changes in gene expression compatible with a more developmentally advanced stage. Additionally, we assessed the relation between electrophysiology and contraction force during cardiomyocyte maturation by analysing these parameters on the same cardiomyocyte.

2. Materials and methods

A detailed description of experimental methods is provided in the Online data supplement.

2.1. hESC and hiPS cell maintenance and differentiation to cardiomyocytes

Both hESC and hiPS lines were cultured on irradiated mouse embryonic fibroblasts (MEFs). Differentiation to cardiomyocytes was as described previously [34]. Briefly, spin embryoid bodies (spinEB) were formed by centrifugation of cells into aggregates in a 96-well format (Greiner) with BSA polyvinylalcohol essential lipids medium (BPEL) [35] together with 35 ng/ml bone morphogenetic protein 4 (BMP4) (R&D), 30 ng/ml Activin A (Miltenyi), 30 ng/ml vascular endothelial cell growth factor (VEGF) (Miltenyi), 40 ng/ml stem cell factor (SCF) (Miltenyi), 1.5 μM CHIR99021 (Axon Medchem). The medium was refreshed on day 3 (Supplementary Fig. 1). SpinEBs started beating between day 7 and 10 of differentiation.

2.2. Culturing and plating of hPSC derived cardiomyocytes

Two commercially available media, Cardiomyocyte medium (CA) and Maturation medium (MM) (available as Pluricyte Medium from Pluriomics BV), were used in a stepwise manner to mature hPSC-CM *in vitro*. CA and MM (containing T3 hormone) are serum-free, defined, nutrient-rich media that were pre-optimized for viability in culture and morphological maturation of hPSC-CMs. Differentiated cardiomyocytes were dissociated at day 10 with 1× TryplE select (Gibco) for 10 min and plated as single cells in BPEL on 0.1% gelatin (Sigma)-coated 6-well plates in order to change from a spinEB to a monolayer culture format. This step is crucial for proper cell attachment to the gelatin lines later on. On day 13, the medium was changed to BPEL or CA (Supplementary Fig. 1). On day 20, one well of each condition was dissociated as described above and seeded on the polyacrylamide gel patterned with 1% gelatin lines, in the respective medium. The medium was refreshed the next day and contraction and electrophysiological characteristics measured three days after plating. In the remaining culture wells the medium was changed to BPEL, CA and MM on day 20 as described in Supplementary Fig. 1. The cells were cultured in the respective media for ten more days. As before, on day 30 the cells were dissociated with 10× Tryple select (Gibco) and seeded on the polyacrylamide gel patterned with 1% gelatin lines, in the respective medium. The cells were refreshed next day and contraction and electrophysiological characteristics measured three days after plating.

2.3. Human fetal cardiomyocyte isolation and culture

Human fetal hearts were collected after elective abortion following individual informed consent, after approval by the Medical Ethical Committee of Leiden University Medical Center. The investigation conforms to the principles outlined in the Declaration of Helsinki. The fetal heart was examined for any apparent anomalies before single cell isolation. The hearts were cut into small pieces and subsequently dissociated in collagenase A (Roche) Calcium-free Tyrode's buffer. To minimize the amount of fibroblasts, the cells were pre-plated on a 10 cm dish for 45 min. The remaining cells were plated directly on the polyacrylamide gel patterned with 1% gelatin lines and kept at 37 °C, 5%CO_2. The medium was refreshed next day and measurements were made three days after plating.

2.4. Patterned polyacrylamide gel fabrication

Patterned polyacrylamide gels were prepared as previously described [36]. Briefly, a polydimethylsiloxane (PDMS) stamp was incubated with 1% gelatin (Sigma) for 1 h. The stamp was used to μcontact-print a pattern of 20 μm wide gelatin lines with 20 μm spacing onto 15 mm coverslips. The polyacrylamide solution was prepared with a final concentration of 0.1% bis-acrylamide (Bio-Rad), 5% acrylamide (Bio-Rad) and 10 mM HEPES pH 8.5 in distilled water, followed by centrifugation for 1 min at 10.000RPM for degassing. 0.006% (m/v) of ammonium persulphate (Sigma–Aldrich) and a 1:1000 dilution of 0.2-μm fluorescent beads (Ex/Em: 660/680 nm - Molecular Probes) were added to the solution and briefly Vortexed. The gel polymerization was initiated with TEMED (Bio-Rad) and 9.2 μl of the final solution was added to a 25 mm coverslip treated with plus Bind-Silane solution (GE Healthcare). The μcontact-printed 15 mm coverslip was placed on top of the drop with the gelatin lines facing the gel. After 20 min of polymerization, the 15 mm coverslip was removed and a 25 mm coverslip was mounted onto a well of a glass bottomed 6-well plate (Mattek), replacing the initial glass. The polymerized gel has a Young's modulus of 5.8 kPa [37].

2.5. Contraction force measurements

The contraction force measurements were performed as previously described [13]. Briefly, using a Leica AF-6000LX microscope with controlled temperature and