Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Biophysical study of human induced Pluripotent Stem Cell-Derived cardiomyocyte structural maturation during long-term culture

Tiago P. Dias [a, b], Sandra N. Pinto [a, c], Juliana I. Santos [a, c], Tiago G. Fernandes [a, b], Fábio Fernandes [a, c], Maria Margarida Diogo [a, b], Manuel Prieto [a, c], Joaquim M.S. Cabral [a, b, *]

[a] iBB - Institute for Bioengineering and Biosciences and Department of Bioengineering, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001, Lisbon, Portugal
[b] The Discoveries Centre for Regenerative and Precision Medicine, Lisbon Campus, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001, Lisbon, Portugal
[c] Centro de Química-Física Molecular and Institute of Nanoscience and Nanotechnology, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001, Lisbon, Portugal

ARTICLE INFO

Article history:
Received 23 March 2018
Accepted 27 March 2018
Available online xxx

Keywords:
Cardiac differentiation
Structural maturation
Long-term culture
Wnt signaling modulation protocol
hiPSC-derived cardiomyocyte microtissues

ABSTRACT

Human induced Pluripotent Stem Cell-derived cardiomyocytes (hiPSC-CMs) have an enormous potential for the development of drug screening and modeling cardiac disease platforms. However, early hiPSC-CMs usually exhibit low structural development, precluding the applicability of these cells. Here, we follow during 120 days the progressive structural maturation of hiPSC-CM microtissues obtained using the Wnt signaling modulation protocol. For this purpose, we designed a user friendly custom-written program to quantify cardiac fiber alignment and sarcomere length. Cardiomyocyte shape, cardiac fiber density and multinucleation were also analyzed. Derived cardiomyocytes showed significant progression in cardiomyocyte fiber density and sarcomere length during the long-term culture, with a peak at day 90 of 40% multinucleated cells. In addition, cardiomyocyte microtissues remained functional with progressive maturation leading to a decrease in the percentage of cTnT positive cells from 59% to 22% at day 120, a value similar to the content present in tissues of the adult left ventricle. These data and the framework that we provide to quantify cardiomyocyte structural features can be important to set new metrics to develop applications for drug screening and disease modeling.

1. Introduction

Cardiovascular diseases are the top cause of death globally with projections from the World Health Organization estimating 23.3 million deaths by 2030. Human induced Pluripotent Stem Cell-derived cardiomyocytes (hiPSC-CMs) have an enormous potential for drug screening and modeling cardiac diseases, allowing the development of platforms to discover new, more efficient, drugs and to screen drugs for hidden cardiotoxicity side effects, reducing overall costs of pharmaceuticals entering the market [1,2]. However, early hiPSC-CMs usually lack the proper structural and electrophysiological maturation to develop relevant applications [3,4]. Furthermore, immature derived cardiomyocytes can show an arrhythmic behavior when used to regenerate cardiac muscle after an ischemic event [5,6]. Therefore, understanding how maturation progresses is essential to develop platforms that can promote/accelerate hiPSC-CMs maturation, allowing these cells to achieve a functionality closer to the one of adult cardiomyocytes.

Commonly, cardiomyocyte maturity is evaluated using methods that assess cardiomyocyte electrophysiology, such as patch clamp [7] micro-electrode arrays [8] or image analysis algorithms [9]. However, electrophysiology characteristics are important but insufficient to assess full cardiomyocyte maturation. Cardiomyocyte contractile ability is only possible due to the multiple sarcomere units integrated in the cell cytoskeleton. In addition, the Troponin complex, present in the thin filaments, is responsible for sensing and inducing calcium mediated responses, prompting sarcomere contraction [10]. Hence, other methods emerged

* Corresponding author. Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001, Lisboa, Portugal.
E-mail address: joaquim.cabral@tecnico.ulisboa.pt (J.M.S. Cabral).

https://doi.org/10.1016/j.bbrc.2018.03.198
0006-291X/

focused on quantifying structural maturation, including the development of algorithms that quantify the alignment of cardiac fibers and sarcomere length [11] or the proposal of cardiac troponin I isoform ratio to quantify cardiomyocyte structural maturation [12]. Nevertheless, these methods can be costly or technically difficult to implement.

Different methods can be employed to differentiate hiPSC into cardiomyocytes [13–16]. The Wnt signaling modulation differentiation protocol has been described to originate high yields and has been widely used to develop multiple platforms [17–19]. Unalike differentiation protocols can originate diverse ratios of the different types of cardiomyocytes [20–22], which can lead to different maturation profiles due to the derived heterogeneous population [4,23]. One method to improve maturation of hiPSC-CMs is the use of long term cultures [24,25]. However, there is a lack of knowledge on the effect of long term cultures in the stepwise structural maturation progression of hiPSC-CM derived using the Wnt signaling modulation protocol.

Here, we show the progressive structural maturation of hiPSC-CM microtissues obtained using the Wnt signaling modulation protocol. Cardiomyocyte maturation was quantified at multiple stages during 120 days in culture by using confocal microscopy and analyzing: (i) cardiomyocyte shape, (ii) alignment, (iii) multinucleation, (iv) cardiac fiber density and (v) quantification of sarcomere and thin filament length. A user friendly custom-written program was developed to quantify cardiac fiber alignment and sarcomere length. Human iPSC-CMs showed a significant progression in most parameters reaching a state of structural maturation closer to adult cardiomyocytes.

2. Materials and methods

2.1. Human induced Pluripotent Stem Cell culture

In this work, the hiPSC cell line iPS-DF6-9-9T.B, purchased from WiCell Bank, was used. This cell line is vector free and was derived from foreskin fibroblasts with a karyotype 46, XY. Maintenance of hiPSC culture was performed using mTeSR1 medium (STEMCELL Technologies) in 6-well tissue culture plates coated with Matrigel (BD Biosciences) diluted 1:30 in DMEM/F12. Cell passaging was performed by incubating cells with 0.5 mM EDTA (Life Technologies) during 5 min.

2.2. Human iPSC-Cardiomyocyte maturation and differentiation

Human iPSC were seeded at a density of 1×10^5 cells/cm^2 and maintained in pluripotent conditions with medium being changed daily. When confluence reached percentages around 95%, hiPSC cardiac differentiation was induced following the Wnt signaling modulation protocol previously described by Lian et al. [26], using 6 μM of the GSK3β inhibitor CHIR99021 (Stemgent) at day 0 and 5 μM of the Wnt signaling inhibitor IWP4 (Stemgent) at day 3 [26].

2.3. Flow cytometry

Microtissues were singularized and fixed using 2% PFA for 20 min at room temperature. Cells were centrifuged and resuspended in 90% cold methanol, incubated for 15 min at 4 °C. Samples were then washed 3 times using a solution of 0.5% BSA in PBS (FB1). Primary antibody Cardiac Troponin T (cTnT) monoclonal mouse IgG antibody (Thermo Scientific, Clone 13-11) was diluted at 1:250 in FB1 plus 0.1% Triton (FB2) and incubated for 1 h at room temperature. Cells were then washed and cell pellet resuspended with the secondary antibody goat anti-mouse Alexa-488 (Life Technologies) diluted 1:1000 in FB2 and incubated for 30 min in the dark. Finally, cells were washed twice and cell pellets were resuspended in 500 μL of PBS and analyzed in a FACSCalibur flow cytometer (Becton Dickinson). Data were analyzed using the software "Flowing Software" (http://www.flowingsoftware.com).

2.4. Immunofluorescence staining

Samples for microscopy were prepared by replating hiPSC-CMs in Matrigel-coated 8-well μ-slides (Ibidi) using EDTA. After 48 h, cells were fixed with 4% PFA for 15 min, washed with PBS and incubated with blocking solution (10% NGS, 0.1% Triton-X in PBS) for 1 h. After incubation, Cardiac Troponin T (cTnT) monoclonal mouse IgG antibody (Thermo Scientific, Clone 13-11) and N-cadherin monoclonal mouse IgM antibody (R&D Clone # 691723) were diluted 1:250 and 1:100, respectively, in staining solution (5% NGS, 0.1% Triton-X in PBS) and incubated for 2 h at room temperature. For staining Actinin, samples were incubated with Sarcomeric α-actinin monoclonal mouse IgG antibody (Sigma, Clone EA-53) at a dilution of 1:200. After washing with PBS, secondary antibodies goat anti-mouse IgG Alexa-488 (LifeTechnologies) and goat anti-mouse IgM Alexa-633 were diluted 1:500 in staining solution and incubated for 1 h at room temperature. Samples were then washed 2 times with PBS, incubated for 2 min with 3 μg/mL of DAPI diluted in PBS, washed again 3 times, and stored at 4 °C.

2.5. Imaging and morphological analysis of hiPSC-derived cardiomyocytes

Confocal Scaning Laser Microscopy (CSLM) and two photon excitation images were acquired using a Leica TCS SP5 confocal inverted microscope (DMI6000) with a 63.3× water-immersion apochromatic objective (1.2 numerical aperture). Alexa-488 excitation was performed using a 488 nm line of an Argon ion laser and fluorescence emission was collected between 500 nm and 560 nm using the tunable system and beam splitter of the Leica TCS SPC5. The same was performed for Alexa-633 using a 633 nm line with fluorescence emission collected between 642 nm and 748 nm. DAPI excitation was achieved using multiphoton excitation and a Ti:sapphire laser (Spectra-Physics Mai Tai BB, 710–990 nm) set to 780 nm as the excitation source. DAPI fluorescence emission was collected between 400 nm and 478 nm. The laser light intensity was controlled by an acoustic-optical filter system. Images were collected with a resolution of 512 × 512 pixels at a scan rate of 100–400 Hz per frame. Morphological analysis were performed using the software Fiji (http://pacific.mpi-cbg.de) and/or a custom-written Matlab program (Fig. S1). Quantitative analysis was performed on a minimum of 10 images from each biological replicate. The custom-written software is freely available upon request from the authors.

2.6. Calcium imaging

Cells were replated on Matrigel-coated 8-well μ-slides (Ibidi) using EDTA. After 48 h, calcium transients were measured using the Fluo-4 Direct Calcium Assay kit (Life Technologies). Fluo-4 was prewarmed at 37 °C and loaded into the cells by adding an equal volume to the culture medium present in the well. Cells were incubated at 37 °C for 30 min.

Samples were measured using the confocal microscope previously described with a dry objective 10.0× magnification (numerical aperture of 0.40), with images obtained at a resolution of 128 × 128 pixels, with a scan speed of 700 Hz, and with frame intervals of 97 msec during 60–120 s. Fluo-4 excitation was performed using a 488 nm line with an Argon ion laser and fluorescence emission was collected (500–650 nm) using a Leica

Please cite this article in press as: T.P. Dias, et al., Biophysical study of human induced Pluripotent Stem Cell-Derived cardiomyocyte structural maturation during long-term culture, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.03.198