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A complete workflow for the differentiation and the dissociation of hiPSC-derived cardiospheres

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ABSTRACT

Cardiomyocytes derived from human induced pluripotent stem cells (hiPSC-CMs) are an invaluable tool for both basic and translational cardiovascular research. The potential that these cells hold for therapy, disease modeling and drug discovery is hampered by several bottlenecks that currently limit both the yield and the efficiency of cardiac induction. Here, we present a complete workflow for the production of ready-to-use hiPSC-CMs in a dynamic suspension bioreactor. This includes the efficient and highly reproducible differentiation of hiPSCs into cardiospheres, which display enhanced physiological maturation compared to static 3D induction in hanging drops, and a novel papain-based dissociation method that offers higher yield and viability than the broadly used dissociation reagents TrypLE and Accutase. Molecular and functional analyses of the cardiomyocytes reseeded after dissociation confirmed both the identity and the functionality of the cells, which can be used in downstream applications, either as monolayers or spheroids.

1. Introduction

Cardiomyocytes derived from human induced pluripotent stem cells (hiPSC-CMs) are an excellent source of patient-specific cardiac cells for pharmacological testing, disease modeling and cardiac tissue engineering. In recent years, remarkable work has been done to identify the conditions needed to recapitulate human cardiac development in vitro. The sequential activation and inhibition of the canonical Wnt pathway was shown to play a critical role for mesoderm specification and terminal cardiomyocyte differentiation, in turn (Burridge et al., 2015; Zhang et al., 2015). This has allowed the production of hiPSC-CMs to become a routine procedure in many cardiovascular laboratories. However, conventional 2D or static 3D culture systems are not suitable for mid- to large-scale production of hiPSC-CMs. Another limitation is the efficiency of cardiac induction, which is subject to operator- and cell line-dependent inconsistencies. This has spurred efforts to move towards cardiac induction in scalable suspension culture systems amenable to a higher degree of automation (Chen et al., 2015; Kempf et al., 2014). Moreover, differentiation in dynamic suspension has the big advantage of providing the cells with a 3D microenvironment that promotes higher physiological maturation, as it allows better intercellular interactions and spatial organization (Pampaloni et al., 2007). It raises, however, the problem of generating spheroids, which are harder to dissociate for downstream applications than conventional

Abbreviations: hiPSC, human induced pluripotent stem cell; CM, cardiomyocyte; BL, BioLevitator; HD, hanging drop; NC, NucleoCounter; LT, LeviTube; Ca, Calcein

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2D monolayers. In this study, we used a benchtop, small footprint suspension bioreactor (BioLevitator; BL) to produce hiPSC-derived cardiospheres. These displayed enhanced maturation compared to those obtained in hanging drops (HD). Combining the dynamic 3D cardiac induction with a novel papain-based dissociation protocol that consistently yields both higher cell number and viability than the broadly-used reagents TrypLE and Accutase, we established a complete workflow for the production of hiPSC-CMs for any downstream applications.

2. Materials and methods

2.1. hiPSCs maintenance

Two hiPSC lines were used: UKBi005, from the European Bank for induced pluripotent Stem Cells and IBMT1, reprogrammed in-house with mRNAs (Stemgent #00-0071) from BJ fibroblasts (ATCC #CRL-2522). Cells were maintained in mTeSR1 medium (Stem Cell Technologies #8580) in 60 mm tissue culture plates coated with growth factor-reduced Matrigel (Corning #356231), according to manufacturer's instructions. Cells were routinely passaged using 0.5 mM EDTA pH 8.0 (Invitrogen #AM9260G) in DPBS without Ca^{2+} and Mg^{2+} ($DPBS^{-/-}$; Gibco #14190144).

2.2. Cardiac induction in 3D

Cardiac fate was induced as previously described (Zhang et al., 2015). Briefly, hiPSCs at 70–80% confluence were harvested using TrypLE Select (Gibco #12563011). Digestion was quenched with 1 volume mTESR1 (Stemcell Technologies #8580) with 10 μM ROCK inhibitor Y27632 (abcam #ab120129). The resulting cell clusters were broken up into single cells with the tip of a pipette. After counting (NucleoCounter NC-200; NC; ChemoMetec, Denmark), cells were diluted in day 0 medium (Table S1) to a concentration of 7.5×10^5 viable cells/mL. Up to 40 mL were transferred to a LeviTube (LT; OMNI Life Sciences #2800005) placed in the BioLevitator (BL; Hamilton, Switzerland). Cells were cultured at 37 °C and 5% CO_2, under continuous rotation at 60 rpm in alternating directions (2 s/direction). After 24 h, the LT was placed on the benchtop to allow the small spheroids to sink to the bottom. Spent medium was replaced with an equal volume of day 1 medium (Table S1). On the following days, medium was in turn replaced with day 2 and day 3 media (Table S1). From day 4 onwards, medium was replaced with TS medium (Table S1) every other day. Cardiac induction in HD was performed as previously described (Zhang et al., 2015) in Perfecta3D 96-well hanging drop plates (Sigma #HDP1096). Medium was exchanged by replacing half of the drop volume twice.

2.3. Enzymatic dissociation of hiPSC-CMs

Cardiospheres were harvested for enzymatic dissociation after 8 or 15 days of cardiac induction. The spheroids from 1 to 2 LTs were washed twice with 2 mM EDTA in $DPBS^{-/-}$. After washing, the spheroids were enzymatically dissociated. Papain dissociation was carried out in a water bath at 37 °C for 30 min. Briefly, the spheroids were taken up in 500 μL $DPBS^{-/-}$ and 500 μL freshly prepared 2× papain solution (Table S2) was added. Digestion was stopped by adding 1 mL freshly prepared 2× stop solution (Table S2). The spheroids were then gently disrupted into a homogeneous suspension by pipetting approximately 20 times with a wide borehole pipette tip. Cells were then washed by adding 5 mL KO-DMEM. For dissociation with Accutase or TrypLE, the spheroids were taken up in 1 mL Accutase (Sigma #A6964) or 1 mL TrypLE Select (Gibco #12563011) and incubated in a water bath at 37 °C for 15 min. The suspension was then homogenized by gently pipetting up and down 5–10 times with a wide borehole pipette tip and the reaction was quenched by adding 5 mL KO-DMEM. Adding the stop solution used in the papain protocol before dilution with KO-DMEM did not have an effect on the dissociation results of Accutase and TrypLE (data not shown). After dissociation, the cells were used directly for analysis or re-seeded in TS medium, either on Matrigel-coated plates at a density of 2.4×10^5 cells/cm^2 or in the BL at a density of 7.5×10^5 cells/mL. The dissociation protocols described for cardiospheres also applied to hiPSC-CMs in monolayers, scaling down the volumes of reagents 5 times.

2.4. Measurement of cell concentration and viability at the NucleoCounter

Cell suspensions from freshly dissociated cardiospheres/monolayers were filtered through a 40 μm cell strainer placed on a 50 mL tube. Undissociated aggregates larger than 40 μm were collected from the strainer that was flipped over onto a new tube and carefully washed with at least 3 mL KO-DMEM. Cells/small clusters in the flow-through were resuspended in 1–3 mL KO-DMEM. The cell concentration in both the undissociated fraction and the flow-through was determined at the NC, following the manufacturer's instructions. To measure both cell concentration and viability, one volume was lysed to count the total number of nuclei and another volume was measured without lysis, allowing only non-viable cells to be stained with DAPI.

2.5. Flow cytometry

To label viable cells for flow cytometry analysis, 1×10^6 freshly dissociated hiPSC-CMs were incubated with 50 ng calcein acetoxymethil (AM; Fisher Scientific #C1430) for 25 min at 37 °C. Propidium iodide (PI; Fisher Scientific #P1304MP) was added at a final concentration of 1 μg/mL to label non-viable cells. Flow cytometry analysis was performed with a FACSAria III (BD).

2.6. Gene expression analysis

Samples were collected for gene expression analysis on days 1 to 7 of cardiac induction or after 2–3 weeks of culture as either cardiospheres or monolayers after dissociation. Briefly, RNA was extracted using the RNeasy Micro kit (Qiagen #74004) following the manufacturer's instructions. The RNA concentration was measured with a Nanodrop 2000 (Thermo Scientific). Reverse transcription was performed with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems #4368814), following the manufacturer's instructions. Gene expression was measured via the 5′-nuclease assay (TaqMan) quantitative PCR (qPCR) using a QuantStudio 7 Flex system (Applied Biosystems) and TaqMan assays (Fisher Scientific #4331182; Table S3). Relative quantification was calculated with the $2^{-\Delta\Delta Ct}$ method using *GAPDH*, *HPRT1* and *GUSB* as endogenous references for normalization.

2.7. Immunocytochemistry

Samples fixed for 15 min in 4.2% PFA (Cytofix, BD #554655) were permeabilized in 0.2% Triton-X-100 (Roth #3051.3) in $DPBS^{-/-}$ for 20 (cell layers) or 30 (spheroids) minutes. After 30 min incubation in blocking solution (BS; Table S2) samples were incubated overnight at 4 °C with primary antibodies against sarcomeric α-actinin (1:200; Sigma #A7811), connexin 43 (1:400; Abcam #ab11370) or desmin (1:500; Abcam #15200). The appropriate Alexa-488 or Alexa-555-conjugated secondary antibodies (Life Technologies #A11034 and #A21422) were diluted 1:1000 in BS and incubated for 1 h at room temperature. DNA was counterstained with DAPI (Fisher Scientific #R37605). Specimens were imaged with a confocal fluorescence microscope (Leica TCS SP8); 200 cells/sample were scored for the expression of the markers tested.