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Development of a scalable suspension culture for cardiac differentiation from human pluripotent stem cells

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ABSTRACT

To meet the need of a large quantity of hPSC-derived cardiomyocytes (CM) for pre-clinical and clinical studies, a robust and scalable differentiation system for CM production is essential. With a human pluripotent stem cells (hPSC) aggregate suspension culture system we established previously, we developed a matrix-free, scalable, and GMP-compliant process for directing hPSC differentiation to CM in suspension culture by modulating Wnt pathways with small molecules. By optimizing critical process parameters including: cell aggregate size, small molecule concentrations, induction timing, and agitation rate, we were able to consistently differentiate hPSCs to >90% CM purity with an average yield of 1.5 to 2 × 10^9 CM/L at scales up to 1 L spinner flasks. CM generated from the suspension culture displayed typical genetic, morphological, and electrophysiological cardiac cell characteristics. This suspension culture system allows seamless transition from hPSC expansion to CM differentiation in a continuous suspension culture. It not only provides a cost and labor effective scalable process for large scale CM production, but also provides a bioreactor prototype for automation of cell manufacturing, which will accelerate the advance of hPSC research towards therapeutic applications.

1. Introduction

Myocardial infarction and heart failure are leading causes of death worldwide. As the myocardium has a very limited regenerative capacity, endogenous cell regeneration cannot adequately compensate for heart damage caused by myocardial infarction. The concept of cell replacement therapy is an appealing approach to the treatment of these cardiac diseases. HPSCs are an attractive cell source for cell replacement therapies because they can be expanded indefinitely in culture and efficiently differentiated into a variety of cell lineages, including cardiac cells. However, current hPSC expansion and differentiation methods rely on adherent cell culture systems that are challenging to scale up to the levels required to support pre-clinical and clinical studies.

Activin/Nodal/TGF-β, BMP, and Wnt signaling play pivotal roles in regulating mesoderm and cardiac specification during embryo development (Arnold and Robertson, 2009; Buckingham et al., 2005; Tam and Loebel, 2007; David et al., 2008; Naito et al., 2006; Ueno et al., 2007; Burridge et al., 2012). Significant progress has been made in the cardiac differentiation process by modulating Activin, BMP, and Wnt pathways, which can efficiently drive differentiation to over 80% purity of CM (Burridge et al., 2014; Kattman et al., 2011; Lian et al., 2012; Yang et al., 2008; Zhang et al., 2012; Zhu et al., 2011). Using an adherent cell culture platform, one study revealed that using 2 small Wnt pathway modulators to sequentially activate and then inhibit Wnt signaling at different differentiation stages of the culture is sufficient to drive cardiac differentiation and generate CM with high purity (Lian et al., 2012). In spite of this, adherent culture systems have limited scalability and are not practical to support the anticipated CM requirements of clinical trials. Alternatively, using an embryoid body (EB) differentiation method, a complex cardiac induction procedure involving stage-specific treatments with growth factors and small molecules to modulate Activin/Nodal, BMP, and Wnt pathways has been reported to be effective in cardiac differentiation in a suspension culture system (Kattman et al., 2011; Yang et al., 2008). However, the process of generating EBs is inefficient, rendering this method impractical for large scale CM production. An additional limitation of these approaches for scale-up application is that both methods are based on the expansion of the hPSCs in adherent culture and the subsequent CM differentiation process in either adherent culture or as EBs. The labor intensiveness and limited scalability of current processes have been the primary bottle

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necks to the large scale production of CM for clinical applications of hPSC-derived CM.

Pre-clinical studies suggest that doses of up to one billion CM will be required to achieve therapeutic benefit after transplantation (Chong et al., 2014; Laflamme and Murry, 2005). In order to meet the current CM demand for pre-clinical studies and the anticipated demand for foreseeable clinical studies, development of a robust, scalable and cGMP-compliant differentiation process for the production of both hPSCs and hPSC-derived CM is essential. Suspension cell culture is an attractive platform for large scale manufacture of cell products for its scale-up capacity. Application of a suspension culture platform to support hPSC growth in matrix-free cell aggregates has been well established (Amit et al., 2010; Krawetz et al., 2010; Olmer et al., 2010; Singh et al., 2010; Steiner et al., 2010; Chen et al., 2012). We previously also reported the development of a defined, scalable and cGMP-compliant suspension system to culture hPSCs in the form of cell aggregates (Chen et al., 2012). With this suspension culture system, hPSC cultures can be serially passaged and consistently expanded. In the present study we adapted our suspension culture system to establish a robust, scalable and cGMP-compliant process for manufacturing CM. We were able to use hPSC aggregates generated in the suspension culture system directly to produce CM with high efficiency and yield in suspension with various scales of spinner flasks. We optimized various critical process parameters including: small molecule concentration, induction timing and agitation rates for differentiation cultures in spinner flasks with scales up to 1 L. In this study, we integrated undifferentiated hPSC expansion and small molecule-induced cardiac differentiation into a scalable suspension culture system using spinner flasks, providing a streamlined and cGMP-compliant process for scale-up CM differentiation and production.

2. Materials and methods

2.1. hPSC suspension cultures

We routinely maintained the hPSCs lines H7 (WA07, WiCell), ESI-017 (BioTime), and a hiPSC line (a gift from Dr. Joseph Wu, Stanford) in the form of cell aggregates in suspension culture as previously described (Chen et al., 2012). Briefly, suspension-adapted hPSCs were seeded as single cells at a density of 2.5–3 × 10^5 cells/mL in 125, 500, or 1000 mL spinner flasks (Corning) containing culture medium (StemPro hESC SFM, Thermo Fisher Scientific, Life Technologies) with 40 ng/mL bFGF (Life Technologies) and 10 μM Y27632 (EMD Millipore). Stirring rates were adjusted to between 50–70 rpm depending on the vessel size and hPSC line. Medium was changed every day by demi-depletion with fresh culture medium without Y27632. Cells were dissociated with Accutase (Millipore) into single cells and passaged every 3–4 days when the aggregate size reached approximately 300 μm. Cell suspension cultures were maintained in 5% CO_2 with 95% relative humidity at 37 °C.

2.2. Calculation of sizes of cell aggregates

Aliquots of aggregates from the suspension cultures were evenly spread in 24- or 6-well plates as necessary to allow adequate separation of aggregates. A minimum of 3 pictures were taken of different areas from the edge to the middle of a well under a microscope. The pictures were analyzed with ImagePro software (Media Cybernetics). At least 200 cell aggregates in the pictures were randomly circled and the sizes of individual cell aggregates and average sizes of selected aggregates were calculated using the software.

2.3. Differentiation of hPSC to CM in suspension

Undifferentiated hESC aggregates generated from suspension cultures were directly used for differentiation in suspension without cell dissociation. Low attachment 6-well plates and spinner flasks in sizes of 125, 500, and 1000 mL were used for differentiation. The differentiation cultures were maintained in 5% CO_2 with 95% relative humidity at 37 °C. RPMI 1640 medium (Life Technologies) with 1× B27® Supplement minus insulin (Life Technologies) was used as the basal medium from CHIR and IWP-4 induction through 2 days post IWP-4 induction. Two days after IWP-4 induction, RPMI 1640 with 1× B27® Supplement (Life Technologies) was used as basal medium. Thereafter, 60–80% of media was change every 2–3 days until cell harvest. Briefly, induction would be initiated on the day hPSC aggregates reached an average size between 160–280 μm. On day 0 of induction, the aggregates were induced with CHIR (Stemgent) at various concentrations. On day 1 the medium was changed to remove the CHIR. On day 2 or 3 IWP-4 (Stemgent) was added at various concentrations for 2 days. On day 4 or 5, the medium was changed to remove IWP-4. After 2 days of IWP-4 induction, basal medium alone were used for further differentiation as described previously.

2.4. Cryopreservation

At harvest, CM aggregates were dissociated with Liberase TH (Roche) at 37 °C for 20–30 min. After washing with PBS, the CM aggregates were further dissociated into single cells with TrypLE (Life Technologies) at 37 °C for 5–10 min. Dissociated single CM at 1–3 × 10^7 cells/mL were cryopreserved with CryoStor CS10 (Biolife Solutions, Inc.) supplemented with 10 μM Y27632 in liquid nitrogen. To carry cells in adherent culture, cryopreserved cells were thawed and plated in 6-well plates coated with Synthemax (Corning) at cell seeding density 1–3 × 10^6 cells per well with 3 mL culture medium RPMI supplemented with B27.

2.5. Flow cytometry

Analysis of the cell surface markers Tra-1-60, SSEA-4, ROR2, PDGFR-α, and CD90: Cells were enzymatically dissociated to single cells, washed in PBS, and counted using a hemocytometer. 2–3 × 10^5 cells were resuspended in FACS buffer composed of 0.5% BSA in PBS and incubated with directly conjugated antibodies for 30 min on ice. After incubation, cells were washed 3 times in FACS buffer and analyzed with an Accuri C6 flow cytometer (BD Biosciences). The antibodies used were: Anti-SSEA-4-PE conjugated (R&D Sysstems), Anti-Tra-1-60-FITC conjugated (BD Biosciences), APC Mouse Anti-Human CD90 (BD Biosciences), mROR2-PE conjugated (FAB20641P, R&D Systems), mPDGFr-alpha-APC conjugated (FAB1264A, R&D Systems).

Analysis of the intracellular marker Oct-4 and cTnT: Cells were enzymatically dissociated to single cells, washed in PBS, and counted using a hemocytometer. Cells were then fixed in 4% PFA in PBS for 10 min at room temperature. Subsequently, cells were washed in PBS, permeabilized in PBS supplemented with 0.1% BSA and 0.1% saponin (permeabilization buffer) for 10 min at room temperature. 2–3 × 10^5 cells were resuspended in permeabilization buffer and incubation with conjugated Oct-4 antibody or cTnT primary antibody for 30 min on ice. Cells stained with cTnT primary antibody were then washed 3 times in permeabilization buffer and incubated with secondary antibody (diluted in permeabilization buffer) for 30 min on ice. After incubation, cells were washed 3 times in FACS buffer and analyzed by Accuri C6. The antibodies used were Anti-Oct3/4-PE conjugated (BD Biosciences) and Anti-Cardiac Troponin T antibody [1C11] (Abcam), and secondary antibody for anti-cardiac Troponin T antibody was R-PE goat anti-mouse IgG (Southern Biotech).

2.6. RNA sequencing

Transcriptome sequencing libraries were constructed with TruSeq RNA Sample Preparation Kit V2 (Illumia) with minor modifications. In brief, 500 ng of total RNA from each sample was used for