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Meticulous optimization of cardiomyocyte yields in a 3-stage continuous integrated agitation bioprocess

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ABSTRACT

Human pluripotent stem cells (hPSCs) can be a renewable source for generating cardiomyocyte (CM) for treating myocardial infraction. In our previous publication, we described an integrated microcarrier-based wave reactor process for the expansion and differentiation of hPSCs to CMs on a rocker based platform. However, this platform is limited in terms of linear scalability and CMs purity. The present study describes ways to overcome these limitations by the use of a stirred scalable platform and incorporation of an additional lactate based purification step which increases CM purity.

Efficient CM differentiation in stirred spinners was achieved by (1) Addition of ascorbic acid (AS) during the differentiation phase which resulted in an increase of 38.42% in CM yield ($0.84 \pm 0.03 \times 10^6$ *vs* $1.17 \pm 0.07 \times 10^6$ CM/mL for cultures without AS and with AS respectively) and (2) Change of agitation regime to a shorter static intervals one (from 66 min off/6 min on (66/6) to 8 min off/1 min on (8/1)) during the first 3 days of differentiation which resulted in 22% increase in CM yield ($1.50 \pm 0.10 \times 10^6$ *vs* $1.23 \pm 0.07 \times 10^6$ CM/mL). The combination of AS addition and change in agitation regime resulted in a production yield of $1.50 \pm 0.10 \times 10^6$ CM/mL which is comparable to that achieved in the rocker platform as described before ($1.61 \pm 0.36 \times 10^6$ CM/mL).

Increase in CM purity was achieved by changing of culture medium to RPMI1640 (without glucose) + 5 mM lactate +0.6 mM AS at day 10 of differentiation which resulted in 44.5% increase in CM purity at day 15. The increase in purity of CMs was due to the death of the non-CM cells (~76% of cell death). It is important to note that in the absence of glucose, lactate was consumed at a rate of 0.01 mmol/10^6 cells/h. Addition of glucose, even in small amounts, during the purification step prevents the process of CM purification, due to the growth of the non-CM cell population.

In summary, hPSC (hESC-HES3 and hiPSC-IMR90) can be efficiently differentiated to CMs in a scalable spinner process which integrates 7 days of expansion ($3.01 \pm 0.51 \times 10^6$ to $3.50 \pm 0.65 \times 10^6$ cells/mL) followed by 10 days of WNT modulated CM differentiation and 5 days of lactate based purification. CM yield of $1.38 \pm 0.22 \times 10^6$ to $1.29 \pm 0.42 \times 10^6$ CM/mL with 72.5 ± 8.35% to 83.12 ± 8.73% cardiac troponin-T positive cells were obtained from these cultures.

1. Introduction

Cardiovascular disorders, one of the major causes of death in the world, can induce progressive loss of contractile heart muscle cells, CM (Dimmeler, 2011). Due to the limited regenerative capacity of the mammalian heart, this ultimately leads to heart failure (Donndorf et al., 2013). While heart transplantation provides the only solution for end-stage cardiovascular disorders, there are serious limitations such as donor supply and immunological incompatibility. As such, substantial research and efforts have been directed towards an alternative cure for cardiovascular disorders: the production of *de novo* CMs as a source for cardiac cell therapy (Zweigerdt, 2007).

It is estimated that cell therapy for a myocardial infarct would require approximately 1 billion CMs (Jing et al., 2008). Due to the limited proliferative capabilities of CMs, human pluripotent stem cells (hPSCs), which include both human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), provide a good source due to their capabilities of unlimited renewal and differentiation to all germ

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layers. Protocols for scale-up and production of *de novo* hPSCs-derived CMs based on the use of aggregate (Dahlmann et al., 2013; Kempf et al., 2015; Niebruegge et al., 2009) or microcarrier (MC) cultures (Lam et al., 2014; Lecina et al., 2010; Ting et al., 2014) in platforms such as spinners, rockers and stirred tank bioreactors have been described in recent years (Chen et al., 2014; Kempf et al., 2016; Lam et al., 2016). However, the multiple steps required for the production process is still problematic and the purity of the differentiated CM is still relatively low (Chen et al., 2011). The general consensus for the optimal process is to establish a minimally manipulated integrated process that combines efficient hPSC expansion, CM differentiation (Chen et al., 2014; Lam et al., 2016) and purification (Ban et al., 2017; Tohyama et al., 2013) in a single reactor (Chen et al., 2014; Kempf et al., 2016).

We have recently shown that the large-scale production of hPSCs-derived CMs can be achieved through the use of suspended 3D MCs on a side to side rocker (wave-type) platform (Ting et al., 2014). Specifically, the platform integrated both hPSC expansion and CM differentiation into a continuous process, resulting in the production of $1.61 \pm 0.31 \times 10^6$ CM/mL (Ting et al., 2014). Recent work in fluid dynamics relating to rocker platform (wave-type reactor) scale-up has shown an increase in shear forces as the volume of the vessel was increased from 2 L to 20 L (Kalmbach et al., 2011). In addition, there was more fluctuation in flow velocity at larger working volumes in comparison to lower working volumes indicating non-homogenous shear stress between scales (Kalmbach et al., 2011).

In comparison, to wave-type reactors on rocker platform, the scale-up of the conventional stirred system has been more thoroughly studied with more correlations established as compared to other systems (Neubauer and Junne, 2016). Specifically, conventional stirred systems have been shown to be linearly scalable as long as the design and geometry of the bioreactors are kept the same (Loffelholz et al., 2014). Also, stirred system bioreactors with larger volumes are more readily available compared to wave reactor systems. Thus, conventional stirred systems are theoretically more appealing as the foundation for a scale-up platform.

Ascorbic acid (AS), vitamin C, is an important water-soluble antioxidant essential for the growth and maintenance of healthy cells in culture. The addition of AS into culture medium has been known to increase CM differentiation efficiencies, by increasing collagen synthesis and by enhancing the proliferation of cardiac progenitor cells *via* the MEK-ERK1/2 pathway, upregulating the late-stage makers of cardiogenesis (Cao et al., 2012; Takahashi et al., 2003).

An additional challenge for the envisioned application of CMs for cell therapy is the requirement of high population purity. Remaining non-CMs present after the differentiation phase may exhibit risk of side effects such as teratoma formation (Hentze et al., 2009). Recently, a methodology based on the unique ability of CMs to metabolize lactate has been established to generate pure CMs post differentiation (Tohyama et al., 2016; Tohyama et al., 2013). In all mammalian cell types, glucose is the main source of energy and anabolic precursor whereby it is converted into pyruvate and/or lactate by glycolysis. Pyruvate is then further utilized in the mitochondrial tricarboxylic acid (TCA) cycle for production of more ATP molecules *via* oxidative phosphorylation (OXPHOS) (Tohyama et al., 2016; Tohyama et al., 2013). CM possesses the ability to efficiently produce energy not only from glucose, but also from fatty acids and lactate *via* OXPHOS. Thus, glucose can be replaced by lactate in the culture medium as the cells' carbon source for CMs, whereas non-CM cells still depend on glucose. This led to the higher CM population from 10% to 99% cells (α-actinin positive) in lactate-containing medium (Tohyama et al., 2013). Burridge et al. (2014) applied the same lactate method to PSCs differentiating as monolayers *via* temporal modulation of the Wnt signaling pathway, using a defined medium with only 4 ingredients – RPMI1640, ascorbic acid, recombinant albumin (BSA) and lactate. Cells were exposed to lactate between differentiation Day 10 and Day 20, enriching the cTnT + CM population from 85% to over 95% of these cells.

In the present study, we integrate a MC suspension based pluripotent stem cell expansion (phase 1), Wnt modulation CM differentiation step (Lian et al., 2013) (phase 2), and CM purification using the lactate-based treatment (Tohyama et al., 2013) (phase 3) into one continuous process in order to improve the efficiency and purity of CM differentiation. Lower CM yields during differentiation previously seen in stirred spinner flask as compared to the wave-type reactor on rocker platform, were corrected with the addition of ascorbic acid to the culture medium during the differentiation phase and applying a shorter static phase intermittent agitation protocol during the first 3 days of differentiation. The addition of lactate in glucose-free purification media enhances the purity of CMs (44.5% increase) by removing the non-CMs in the cultures.

2. Materials and methods

2.1. Expansion and maintenance of hESC and hiPSC in monolayer cultures

hESC (HES-3) ([46 X, X]; ES Cell International) and hiPSC (IMR-90) ([46 X, X]; provided by James Thomson (Yu et al., 2007)) with normal karyotypes were cultured in mTeSR™1 (Stemcell Technologies) medium on tissue culture plates coated with Geltrex® (ThermoFisher Scientific) at 37 °C in a 5% CO_2 incubator, following a previously described protocol (monolayer cultures) (Choo et al., 2006).

2.2. Maintenance of hESC and hiPSC in MC static suspension cultures

Cytodex 1 MCs (GE healthcare; 1 mg dry weight/mL) were coated with Geltrex® (ThermoFisher Scientific) for all cultures used. Procedures for preparation, sterilization, and coating of MCs are previously described (Chen et al., 2011).

For initiation of MC cultures, cells from monolayer cultures (MNL) were detached using Dispase (ThermoFisher Scientific) into small clumps (100 μm) and seeded at 2×10^5 cells/mL into ultra-low attachment (ULA) six-well plates (Corning) containing 5 mg MCs in 5 mL of mTeSR™1 medium (StemCell Technologies) per well. The MC cultures were then maintained at 37 °C in 5% CO_2 incubator for 7 days expansion, with 80% of the medium changed daily. Thereafter, cell passage was done by mechanical dissociation of the cell/MC aggregates into small clumps (1–3 Cytodex MCs per clump). 2×10^5 cells/mL of cell/MC were then transferred into new six-well plates containing 5 mg MCs in 5 mL of medium per well (Ting et al., 2014).

2.3. Expansion of hESC and hiPSC in MC spinner or rocker flask cultures (Phase 1)

MC spinner flask cultures were operated in a procedure similar to the one previously described (Chen et al., 2011). Briefly, cells obtained from MC static suspension cultures aforementioned were seeded into 125 mL disposable spinner flasks (Corning) containing 1 mg dry weight/mL coated with Geltrex®, at a cell concentration of 2×10^5 cells/mL. Spinner cultures were maintained static for one day and minimal stirring (25–30 rpm) was initiated on the second day.

MC rocker cultures were operated in a procedure as described previously (Ting et al., 2014). Briefly, cells obtained from MC static cultures were seeded at a concentration of 4×10^5 cells/mL into T-25 ULA flasks (Corning) (containing 15 mL medium and 30 mg MCs) (refer to schematic Fig. 1A). Then, the flasks were placed on laboratory rocker (ThermoFisher Scientific) under static conditions for 1 day. Thereafter, 15 mL medium was added into the flasks and the cultures were agitated at rocking rate of 30 oscillations/min at a tilt angle of 12°. Rocking speed and tilt angle were predetermined to ensure even suspension of aggregates.

2.4. Differentiation of hESC and hiPSC in MC spinner or rocker flask cultures (Phase 2)

Pluripotent cell/MC aggregates obtained from the expansion phase in MC spinner or MC rocker cultures were washed three times by