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An intermittent rocking platform for integrated expansion and differentiation of human pluripotent stem cells to cardiomyocytes in suspended microcarrier cultures

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Abstract The development of novel platforms for large scale production of human embryonic stem cells (hESC) derived cardiomyocytes (CM) becomes more crucial as the demand for CMs in preclinical trials, high throughput cardio toxicity assays and future regenerative therapeutics rises. To this end, we have designed a microcarrier (MC) suspension agitated platform that integrates pluripotent hESC expansion followed by CM differentiation in a continuous, homogenous process.

Hydrodynamic shear stresses applied during the hESC expansion and CM differentiation steps drastically reduced the capability of the cells to differentiate into CMs. Applying vigorous stirring during pluripotent hESC expansion on Cytodex 1 MC in spinner cultures resulted in low CM yields in the following differentiation step (cardiac troponin-T (cTnT): 22.83 ± 2.56%; myosin heavy chain (MHC): 19.30 ± 5.31%). Whereas the lower shear experienced in side to side rocker (wave type) platform resulted in higher CM yields (cTNT: 47.50 ± 7.35%; MHC: 42.85 ± 2.64%). The efficiency of CM differentiation is also affected by the hydrodynamic shear stress applied during the first 3 days of the differentiation stage. Even low shear applied continuously by side to side rocker agitation resulted in very low CM differentiation efficiency (cTnT < 5%; MHC < 2%). Simply by applying intermittent agitation during these 3 days followed by continuous agitation for the subsequent 9 days, CM differentiation efficiency can be substantially increased (cTNT: 65.73 ± 10.73%; MHC: 59.73 ± 9.17%). These yields are 38.3% and 39.3% higher (for cTnT and MHC respectively) than static culture control.

During the hESC expansion phase, cells grew on continuously agitated rocker platform as pluripotent cell/MC aggregates (166 ± 88 × 10^5 μm^2) achieving a cell concentration of 3.74 ± 0.55 × 10^6 cells/mL (18.89 ± 2.82 fold expansion) in 7 days. These aggregates were further differentiated into CMs using a WNT modulation differentiation protocol for the subsequent 12 days on a rocking platform with an intermittent agitation regime during the first 3 days. Collectively, the integrated MC rocker platform produced 190.5 ± 58.8 × 10^6 CMs per run (31.75 ± 9.74 CM/hESC seeded). The robustness of the system was

Abbreviations: cTnT, cardiac troponin-T; CM, cardiomyocyte; EB, embryoid body; hESC, human embryonic stem cells; hiPSC, HUMAN induced pluripotent stem cells; MC, microcarrier; MHC, MYOSIN heavy chain

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demonstrated by using 2 cells lines, hESC (HES-3) and human induced pluripotent stem cell (hiPSC) IMR-90. The CM/MC aggregates formed extensive sarcomeres that exhibited cross-striations confirming cardiac ontogeny. Functionality of the CMs was demonstrated by monitoring the effect of inotropic drug, Isoproterenol on beating frequency.
In conclusion, we have developed a simple robust and scalable platform that integrates both hESC expansion and CM differentiation in one unit process which is capable of meeting the need for large amounts of CMs.

Introduction

Cardiovascular disease is one of the major causes of death in the United States, accounting for 31.9% of all deaths in 2010 (Go et al., 2014). The discovery that functional cardiomyocytes (CM) can be obtained from human embryonic stem cells (hESC) or human induced pluripotent stem cells (hiPSC) (Itskovitz-Eldor et al., 2000; Zwi et al., 2009), led to the increase in research directed toward the utilization of these CMs as a potential source for treating heart diseases by cell therapy (Burridge et al., 2012). As such, substantial effort has been made to improve CM differentiation efficiencies (Burridge et al., 2011; Lian et al., 2012) by developing multiple differentiation protocols with some capable of producing more than 90% pure populations of CMs (Burridge et al., 2012; Chen et al., 2014; Xu, 2012) in lab-scale 2D platforms. However, due to the large quantities of CMs needed for cell therapy, development of methodologies for 3D suspended scalable production platforms still remain a major challenge (Burridge et al., 2012; Chen et al., 2014).

Due to the limited proliferative capability of CMs, large scale production of CMs is based on the expansion of pluripotent hESC followed by a CM differentiation step (Chen et al., 2014). Scaling up of hESC expansion in monolayer (MNL) culture platform is problematic mainly due to surface area limitation. One of the approaches to overcome this hurdle is the use of suspended 3D microcarriers (MC) which can provide large surface area per culture volume (Oh et al., 2009; Chen et al., 2011). Oh et al. demonstrated that hESC can be expanded to densities of 3.5×10^6 cells/mL (18 fold expansion) in MC spinner flask culture while retaining hESC pluripotency, ability to differentiate into the 3 germ layers, and normal karyotypes (Oh et al., 2009). In comparison, parallel hESC expansion on MNL platforms achieved densities of 0.8×10^6 cells/mL (4 fold expansion) (Oh et al., 2009).

Induction of CM differentiation can be achieved by using small molecules (Lian et al., 2013; Graichen et al., 2008; Minami et al., 2012) or growth factors (Sa and McCloskey, 2012; Kattman et al., 2011). High differentiation efficiencies can be achieved by both methods, however, the small molecule approach provides a distinct advantage in terms of cost and reproducibility and thus has the ability to be amenable to GMP standards (Kirouac and Zandstra, 2008). One example of a highly efficient small molecule CM differentiation protocol is reported by Lian et al. (2012, 2013). In this protocol, CM differentiation is initiated by WNT pathway activation at day 0 using CHIR99021 (CHIR) or 6-bromoindirubin-3′-oxime (BIO) and is followed by its repression at day 3 using inhibitors, IWP-2 or IWP-4. Differentiation efficiencies of up to 98% cardiac troponin-T positive cells were reported for MNL cultures.

CM differentiation in 2D MNL cultures can achieve high differentiation efficiency but is limited in scalability (Rowley et al., 2012). In comparison, protocols using 3D suspended embryoid body (EB) cultures achieve lower differentiation efficiencies (Chen et al., 2014) but have the ability to be scaled up volumetrically (Niebruegge et al., 2008). Generating EB structures is problematic though, since it requires extensive cell handling as pluripotent cells need to be dissociated and re-aggregated to the appropriate size, in order to create efficient EBs for further differentiation. This causes difficulties in maintaining reproducibility and cell viability, thus limiting its ability to be developed into an efficient and robust bioprocess (Placzek et al., 2009).

In the present study, we attempt to integrate MC suspension based pluripotent hESC expansion followed by CM differentiation using the WNT modulation protocol (Lian et al., 2012) as a continuous process in order to increase the efficiency of CM differentiation. Hydrodynamic shear stress applied during culture agitation was found to drastically affect CM yields. Expansion of HES-3 in the vigorously agitated MC spinner culture resulted in 38.1% reduction of CM yield in the following differentiation step, as compared to static control MC cultures. While hESC expanded in the gentler rocker platform achieved an increase of 32.1% CM yield. Moreover, agitation applied during the first 3 days of the differentiation process was also observed to have an inhibitory effect on CM generation. Thus, an efficient CM production platform was developed in which pluripotent cells were expanded in continuously agitated rocker culture followed by differentiation during which intermittent rocking was applied only on days 1–3 followed by continuous agitation until day 12. This MC based platform provides 18.89 ± 2.82 folds of cell expansion and CM differentiation efficiencies of 31.75 ± 9.74 CM/hESC having 65.73 ± 10.73% and 59.73 ± 9.17% expression levels for cTnT and MHC respectively. In total, this platform was capable of producing $190.5 \pm 58.8 \times 10^6$ CMs per run. The robustness of the integrated MC rocker platform was further demonstrated with an additional human induced pluripotent stem cell (hiPSC) line, achieving 19.56 ± 0.44 CM/hiPSC. The CMs obtained from this process were examined for structural and functional properties through immunohistology and exposure to inotropic substance, Isoproterenol, respectively. The new method offers a simple means for the scalable production of CMs in large quantities through the integrated bioprocess of cell propagation and differentiation.