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 × 10⁵ μm²) achieving a cell concentration of 3.74 ± 0.55 × 10⁶ 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 × 10⁶ 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.

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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 (Burr ridge et al., 2012). As such, substantial effort has been made to improve CM differentiation efficiencies (Burr ridge et al., 2011; Lian et al., 2012) by developing multiple differentiation protocols with some capable of producing more than 90% pure populations of CMs (Burr ridge 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 (Burr ridge 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 $3.5 \times 10^6$ 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 $0.8 \times 10^6$ 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 $18.89 \pm 2.82$ folds of cell expansion and CM differentiation efficiencies of $31.75 \pm 9.74$ CM/hESC having $65.73 \pm 10.73$% and $59.73 \pm 9.17$% 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 $19.56 \pm 0.44$ 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.
Materials & methods

Expansion and maintenance of hESC and hiPSC in ML cultures

hESC (HES-3) ([46 X, X]; ES Cell International) and human induced pluripotent stem cell (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 Matrigel™ (BD) at 37 °C in 5% CO₂ incubator, following a previously described protocol (Choo et al., 2006).

Expansion of hESC and hiPSC in MC cultures

Fig. 1A presents the different MC cultures expansion process used in this work: (i) MC static culture, (ii) MC rocker culture, and (iii) MC spinner culture. All cultures used Cytodex 1 MCs (GE healthcare) at a concentration of 1 mg dry weight/mL coated with Matrigel™ (BD). Protocols for preparation, sterilization and coating of MCs are previously described in Chen et al. (2011). The cultures were maintained for 7 days in mTeSR™1 (Stemcell Technologies) medium, at 37 °C in 5% CO₂ incubator with 80% of the medium refreshed daily.

For initiation of MC static cultures, cells from MNL cultures were enzymatically detached (Dispase; Invitrogen) into small clumps (100 μm) and seeded at 2 × 10⁵ cells/mL into ultra-low attachment six-well plates (Corning) containing 5 mg MCs in 5 mL of mTeSR™1 per well. After 7 days of propagation, cell passage was done by mechanically dissociation of the cell/MC aggregates into small clumps (1–3 Cytodex 1 MCs per clump), which were transferred at 2 × 10⁵ cells/mL into new six-well plates containing 5 mg MCs in 5 mL of medium per well. These static MC cultures were maintained for up to 10 passages without losing their pluripotency (Fig. 1A) (Oh et al., 2009).

MC spinner flask cultures

MC spinner flask cultures were operated in a procedure similar to the one previously described by Chen et al. (2011). Briefly, cells obtained from MC static cultures were seeded at cell
concentration of $4 \times 10^5$ cells/mL into 100 mL spinner flasks (BellCo) containing 25 mL medium and 50 mg MCs. Spinner cultures were maintained static for one day. On the second day, 25 mL of mTeSR™1 was added and stirring was initiated at 25 rpm. This is the minimal agitation speed that ensures even suspension of aggregates.

**MC rocker cultures**

In MC rocker cultures, cells obtained from MC static cultures were seeded at concentration of $4 \times 10^5$ cells/mL into ultra-low attachment (ULA) T-25 flasks (Corning) (containing 15 mL medium and 30 mg MCs) which were placed on laboratory rocker (Thermo Scientific). After one day of static conditions, 15 mL medium was added and the cultures were agitated at rocking rate of 30 oscillations/min and at a tilt angle of 12°. Rocking speed and tilt angle was pre-determined to ensure even suspension of aggregates.

**Differentiation of hESC and hiPSC on MCs**

Pluripotent cell/MC aggregates obtained from MC static, MC spinner and MC rocker cultures were differentiated to CMs in (I) static six-well ULA plate (MC static culture) and (II) ULA T-25 flask on laboratory rocker (MC rocker culture). Aggregates were washed ($\times$3) by refreshing 80% of the original medium volume with RPMI 1640 medium supplemented with B27-insulin (Invitrogen). Aggregate suspensions were then diluted and seeded at a concentration of $1 \times 10^6$ cells/mL into three ULA T-25 flask or six-well ULA plates with a total medium volume of 30–33 mL/flask or 5 mL/well respectively. Differentiation was done at 37 °C with 5% CO$_2$ according to a protocol previously described by Lian et al. (2013) and is summarized in Fig. 1B. Briefly, CHIR99021 (CHIR) in RPMI 1640 supplemented with B27-insulin (Invitrogen) was added at concentration of 18 μM for the first 24 h and then removed via medium change (day 0 to day 1). On day 3 of differentiation, cells were treated to 5 μM IWP-2 (Stemgent) in RPMI 1640 supplemented with B27-insulin. IWP-2 was removed via medium change on day 5 and cells were maintained in RPMI 1640 supplemented with B27-insulin thereafter. On day 12, cultures were harvested for analysis. In some rocking cultures, intermittent agitation (6 min on 66 min off) was introduced in the first 1, 2 and 3 days of differentiation to evaluate shear stress on cardiogenesis. Results were quantified by comparing the yields of CM/hESC seeded which refers to the amount of CMs generated per hESC seeded at the beginning of the hESC expansion or differentiation phase.

The integrated process that includes hESC propagation, the transition from hESC expansion to CM differentiation and harvesting the CMs at day 12 of differentiation is referred to as a "production run".

**Separation of microcarriers from cardiomyocyte cultures**

CM/MC aggregates, harvested on day 12 of differentiation, were enzymatically dissociated into a single cell/MC suspension using Tryple™ (1x; 15–20 min) (Invitrogen). The single cell/MC suspension was subsequently passed through a 40 μm cell strainer (Becton Dickinson) to separate the cells from the MCs.

**Western blotting**

Cells were lysed with lysis buffer (Cell Signaling Technology) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma), 1× protease inhibitor cocktail (Nacalai Tesque) and 1× phosphatase inhibitor cocktail (Roche). Proteins (50 μg/well) were separated on 4–12% gradient Bis–Tris NuPage gels (Life Technologies) and blotted on methanol-activated PVDF membrane (Bio-rad). Thereafter, blocked membranes were incubated with a specific goat anti-human SMAD7 antibody (1:500; Genetex) overnight at 4 °C in the same blocking buffer. Membranes were washed with 0.1% Tween-20 (Bio-rad) in Tris-buffered saline (1st BASE), and incubated with anti-rabbit-HRP conjugate (1:10,000; Dako) for 90 min at room temperature. Immunocomplexes were visualized with an Immobilon Western Chemiluminescent HRP substrate (Millipore) on a chemiluminescence detection imaging instrument (LAS500; GE Healthcare).

**qRT-PCR**

Total RNA was isolated from the cells ($\sim 5 \times 10^6$) using both Trizol (Invitrogen) and RNeasy Mini Kit (Qiagen) following the supplier’s protocol. Reverse transcription was carried out with 1 μg total RNA using Maxima First Strand cDNA synthesis kit (Fermentas). Real-time PCR was performed by applying a standard two-step amplification protocol on an ABI 7500 system (Applied Biosystem) to detect mRNA expression. Relative expression values were obtained by normalizing $C_T$ values of the tested genes to the $C_T$ values of the housekeeping gene GAPDH using the $\Delta\Delta C_T$ method. Primer sequences are provided in Supplementary Table 1.

**Immunohistology**

CM cell aggregates were harvested on day 12 of differentiation, washed with phosphate-buffered saline (PBS) with Ca$^{2+}$/Mg$^{2+}$, and fixed with fixing reagent A (Invitrogen). Cryo-sectioned slides were washed twice with PBS, permeabilized and blocked by 0.1% Triton X-100 and 10% goat serum respectively. The following antibodies were used: anti-MYL-2A (MLC-2A; Synaptic Systems), anti-SA (Sigma), and anti-tropinin-T (Thermo Scientific). Nuclear staining was done using Slow Fade Glow with DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen). The fluorescence was observed using a Nikon Ti-E fluorescence microscope coupled with Nikon imaging software, NIS elements.

**Flow cytometry**

Aggregates were dissociated into single cells with Tryple™ (1x) (Invitrogen) and the cell/MC suspension was filtered through a 40 μm cell strainer (Becton Dickinson) to remove the MCs. Subsequently, cells were fixed and permeabilized (Invitrogen) according to manufacturer’s directions and incubated with primary antibodies, anti-myosin heavy chain (anti-MHC; MF20, 1:200; Developmental Studies Hybridoma Bank) and anti-
troponin-T (anti-cTnT; 1:200; Thermo Scientific) for CM differentiation efficiency assessment and OCT 4 (1:100; R&D Systems), mAB84 (1:20) (Choo et al., 2008) and SSEA4 (1:100; BioLegend) for hESC pluripotency assessment. Alexa Fluor 647® goat anti-mouse (Invitrogen) was used as the secondary antibody. All incubations were conducted at room temperature for 30 min. Fluorescent measurements were done using flow cytometer (GUAVA, Millipore).

**Metabolite analysis**

Metabolite analysis (glucose, lactate, ammonium and glutamine) were determined by Bioprofile 100 plus (NOVA). Methods for calculating specific metabolite consumption and waste product production rates are described previously by Chen et al. (2010).

**Toxicology assay**

Contracting CM aggregates were transferred into ultra-low attachment 96-well plates (Corning). Cardiac inotropic substance, Isoproterenol (Sigma-Aldrich), was added at 1 μM concentration. Videos were recorded before and after addition of cardiac toxic drugs and were analyzed for beating frequency changes using an imaging platform (TVAMS) previously described by Ting et al. (2014).

**Cell count**

Aggregates cultures were swirled to form a uniform mixture then sampled and lysed for total and viable cell count using a nuclei count method with DAPI using NucleoCounter NC-3000 (Chemometec) according to the manufacturer’s instructions.

**Aggregate size measurement**

Aggregate sizes were evaluated by measuring the two-dimensional area of the microscopic images of the aggregates (n > 50) using Nikon Ti-E phase contrast microscope coupled with Nikon imaging software, NIS elements.

**Statistics**

For comparison between two data sets, significance was calculated by Bonferroni corrected Student’s t-test. For comparison between multiple data sets, significance was calculated by Bonferroni corrected one-way ANOVA test. Error bars indicated on figures represent +/- standard deviations of at least 3 repetitions.

**Results**

hESC MC culture expansion phase: Pluripotent cell growth in spinner or rocking systems and the effects on subsequent CM differentiation efficiency

Large quantities of pluripotent hESC can be obtained in scalable agitated MC culture (Wang et al., 2005). However, the shear stress introduced by agitation is known to affect cell viability, cell pluripotency and most importantly, the ability of cells to differentiate (Leung et al., 2011; Earls et al., 2013). This for reason, MC cultures with three levels of shear rates: (i) MC static culture, and (ii) MC Rocker culture (approximately 0.1 dyn/cm² as measured in previous study (Oncul et al., 2010)) (iii) MC spinner culture (approximately 1.1 to 2.4 dyn/cm² as measured in previous studies (Santos et al., 2011; Sucosky et al., 2004)) were assessed for hESC expansion, hESC pluripotency and subsequent CM differentiation efficiency. The flow of these experiments is presented in Fig. 1A, dotted line “—”. Both agitated cultures (MC spinner culture and MC rocker culture) demonstrated shorter doubling times and higher cell yields than the MC static culture control. Doubling times of 34 ± 4 and 37 ± 7 h for MC spinner culture and MC rocker culture respectively as compared to 102 ± 7 h for MC static culture and cell yields of 2.95 ± 0.20 × 10⁶ and 3.74 ± 0.55 × 10⁶ cells/ mL for MC spinner culture and MC rocker culture respectively as compared to 1.44 ± 0.17 × 10⁶ cells/mL MC static culture (Fig. 2A). Cell viability was not affected (above 85%) in all 3 systems (MC static, MC rocker and MC spinner). The metabolic activity of the cells was similar in all systems (qGlc = 0.36 ± 0.03 mmol/10⁹ cells/h; qLac = 0.74 ± 0.07 mmol/10⁹ cells/h; qGln = 0.032 ± 0.002 mmol/10⁹ cells/h; qAmm = 0.025 ± 0.002 mmol/10⁹ cells/h; Ybac/Glc = 2.06 ± 0.06; YKm/Gln = 0.79 ± 0.12). However, the mode of cell propagation had a significant effect on the shape and size of the cell-MC aggregates. In static cultures, very large (~180 × 10² μm²) non-uniform amorphous aggregates were seen (Fig. 2C). In the agitated cultures, more defined uniform sized spherical shapes were generated (Fig. 2C), with the gentler agitated MC rocker culture system resulting in larger sized aggregates (166 ± 88 × 10² μm²) as compared to the more vigorous MC spinner cultures (85 ± 42 × 10² μm²) (Figs. 2B and C). Variance in aggregate sizes was also large in MC rocker cultures in comparison to MC spinner cultures. The different levels of shear did not affect pluripotency with expression levels of OCT4, mAB84 and SSEA4 above 90% in all the cultures at day 7 of growth.

The aggregates from the 3 systems were transferred to six-well plates, differentiated for 12 days in static conditions using the WNT protocol (Fig. 1B), and subsequently evaluated for cell yields and differentiation efficiency. Cell expansion during the differentiation process was similar in all system (cell fold expansion between 1.28 and 1.57 and cell viability above 90%). However, higher expression levels of cTnT (36.95 ± 2.07% and 47.50 ± 7.35%) and MHC (31.58 ± 9.64% 42.85 ± 2.64%) was obtained in cultures which were expanded in static and rocking systems respectively as compared to those expanded in the MC spinner cultures (22.83 ± 2.56% (cTnT) and 19.30 ± 5.31% (MHC)) (Fig. 2D). Collectively, the results confirm that pluripotent cell expansion in the MC rocker culture platform is the method of choice for further exploration, since high cell yields were obtained. More importantly, the following CM differentiation efficiency was not affected by the agitation applied during the expansion phase.

hESC CM differentiation phase: effects of agitation on CM differentiation efficiency

Preliminary experiments (not shown) demonstrated that cell/MC aggregates obtained from static or agitated cultures
treated by the WNT differentiation protocol regime (Fig. 1B) for 12 days under continuous agitation (rocker) does not exhibit significant CM differentiation (cTnT expression below 5% as compared to 47.50 ± 7.35% in static cultures) indicating that the shear stress during agitation inhibits CM differentiation process. We therefore examined whether applying intermittent agitation (6 min on/66 min off) for 1, 2 or 3 days followed by continuous agitation might improve cardiogenesis in comparison to continuous agitation from day 0.

HES-3 cells expanded on the MC rocker platform for 7 days were further differentiated for 12 days (T-25 flask) on the rocker platform (The flow of the experiment is demonstrated in Fig. 1A, dotted line “—”). Intermittent agitation was applied for 1, 2 or 3 days followed by continuous agitation. Static and continuous agitated cultures were used as positive and negative controls respectively. As expected, continuous agitation for 12 days utterly inhibits the CM differentiation process (cTnT < 5%; MHC < 2%); while in static MC cultures, high expression levels of cTnT (47.50 ± 7.35%) and MHC (42.85 ± 2.64%) were achieved (Fig. 3A). It was found that maintenance of intermittent agitation for the first 3 days is essential for efficient CM differentiation. High differentiation efficiency (65.73 ± 10.73 and 59.73 ± 9.17% expression of

![Figure 2](image_url)
cTnT and MHC markers respectively) was achieved as compared to 5.43 ± 3.82% cTnT and 8.08 ± 4.45% MHC for cultures in which intermittent agitation was maintained for only 1 or 2 days (Fig. 3A). Intermittent agitation for just 1 or 2 days did not improve cardiogenesis.

Cell expansion during the differentiation process was higher in all agitated cultures as compared to static conditions (2.31 ± 0.34 × 10⁶ vs. 1.54 ± 0.12 × 10⁶ cells/mL) again probably due to diffusional limitations in static cultures (Fig. 3A). Aggregates generated in the intermittent agitated (3 days) MC rocker culture system were larger in size as compared to aggregates generated in continuous agitated MC rocker culture system (148.94 ± 52.92 × 10⁵ μm² vs. 78.09 ± 37.42 × 10⁵ μm² respectively) (Fig. 3B).

In summary, the results indicate that the MC rocker culture platform that incorporates hESC expansion followed by differentiation step with intermittent agitation regime for only the first 3 days of differentiation, can be used as an efficient, one unit, integrated system for CMs production.

Characterization of the integrated platform: reproducibility of cell growth, metabolism of glucose and glutamine, robustness, and gene expression during differentiation

Multiple production runs, which includes both the hESC expansion and CM differentiation steps, was conducted on the MC rocker platform to further analyze for reproducibility of cell growth, CM yields, metabolism of glucose and glutamine, universality, and gene expression during differentiation.

During hESC expansion, cell yields of 3.74 ± 0.55 × 10⁶ cells/mL with 96.10 ± 3.44% viability were achieved (n = 4) (Table 1A). At the end of the expansion phase, 166 ± 88 × 10⁵ μm² cell/MC aggregates were obtained and pluripotent markers were above 90%.

During the first day of differentiation (after addition of CHIR99021), cell viability dropped to 71.66% and consequently during the next day, a drop in cell density was observed (from 1 × 10⁶ cells/mL to 4.55 × 10⁵ cells/mL) (Fig. 4A). The drop in cell viability was probably due to the

Figure 3  Effects of agitation during the first 3 days of differentiation on CM yields. Pluripotent HES-3 cell/MC aggregates obtained from rocker cultures were differentiated in intermittently agitated MC rocker for the first 1, 2 or 3 days followed by continuous agitation for the following 11, 10, and 9 days respectively. Continuous agitated MC rocker cultures and static MC cultures were operated in parallel as controls. (A) Cell yield and expression levels cardiac troponin-T (cTnT) and myosin heavy chain (MHC) (n = 3, *p < 0.001, Bonferroni corrected) and the (B) shape of cell/MC aggregates were recorded after 12 days of differentiation.
abrupt change from hESC culture medium (mTeSR™) to differentiation medium which lacks insulin (as insulin inhibits differentiation (Freund et al., 2008)). However, subsequent cell growth between days 3–12 resulted in overall 2.45 ± 0.12 fold expansion with cell density of 2.45 ± 0.12 × 10⁶ cells/mL achieved (n = 6; 99.56 ± 0.20% viability). Expression levels of cTnT and MHC were 65.73 ± 10.73% and 59.73 ± 9.17% respectively (Table 1B).

The entire integrated process which took 19 days (7 days hESC expansion and 12 days differentiation) resulted in 190.5 ± 58.8 × 10⁶ CMs/production run and 31.75 ± 9.74 positive cTnT CMs per hESC seeded at the beginning of the expansion phase (Table 1C).

During the differentiation phase, cells exhibit longer doubling time (131.46 ± 33.6 h vs 37 ± 7 h) and lower metabolic activity in comparison to the expansion phase. Specifically, glucose consumption (0.10 ± 0.01 mmol/10⁹ cells/h) and lactate production (0.10 ± 0.02 mmol/10⁹ cells/h) rates as well as specific glutamine consumption (0.06 ± 0.01 mmol/10⁹ cells/h) and ammonium production rates (0.04 ± 0.01 mmol/10⁹ cells/h) were lower compared to these metabolic parameters measured during the expansion phase (0.38 ± 0.18; 0.77 ± 0.38; 0.31 ± 0.10 × 10⁻¹; 0.27 ± 0.06 × 10⁻¹ mmol/10⁹ cells/h respectively). It is important to note that glucose consumption is more aerobic during the differentiation phase in comparison to the expansion phase (YLac/Glc 0.96 ± 0.08 vs. 1.99 ± 0.02 respectively) while glutamine metabolism is similar (YAmm/Gln = 0.81 ± 0.13).

During the first 3 days of CM differentiation, a down regulation of pluripotent markers, OCT4 and Nanog were observed (Fig. 4B). This was followed by an up regulation of mesoderm marker, T-bra, and cardiac progenitor markers, Nkx2.5 and ISL-1 during the next 3 days (Fig. 4B). During the last 5 days of differentiation, up regulation of the CM marker, MHC, was observed (Fig. 4B).

A process for the separation of CMs from the MCs was also developed. The CM/MCs aggregates generated using the integrated platform was separated using an enzymatic dissociation process and a 40 μm cell strainer. Cell viability and cell recovery for this cell separation process was 88.33 ± 0.02% and 90.56 ± 2.84% respectively.

The robustness of the integrated platform was validated by using an additional hiPSC cell line (IMR-90) (Table 1a–c). Similar to HES-3 cultures, a total of 117.4 ± 2.7 × 10⁶ cTnT positive CMs per production run was produced with a purity of 46.65 ± 5.73% and a yield of 19.56 ± 0.44 cTnT positive CMs per hiPSC seeded were obtained (Table 1C).

### Structural and functional characterization

CM structure of differentiated cell/MC aggregates was evaluated by immunofluorescence (Fig. 5A). The aggregates were stained positive for cardiac troponin-T (cTnT), myosin light chain (MYL-2A) and sarcomeric α-actinin (SA), showing organized sarcomere structures. Interestingly, there was a higher expression of sarcomeric α-actinin (SA) and actin in regions surrounding the MCs (Fig. 5B). The functionality of the produced CMs was evaluated by monitoring the effect of inotropic drug, Isoproterenol (1 μM), on beating frequency using an imaging system previously developed by Ting et al. (2014). The addition of the drug resulted in a 62% increase in beating frequency (from 0.75 ± 0.11 to 1.29 ± 0.17 Hz) (Fig. 5C). The long term stability of these beating cell/MC aggregates was then demonstrated by maintaining the aggregates in RPMI 1640 supplemented with B27-insulin.
medium for 7 months, these aggregates displayed similar beating frequencies as day 12 aggregates (0.75 ± 0.11 vs. 0.79 ± 0.14 Hz respectively) (Fig. 5C).

Discussion

We present a suspended MC based integrated rocker platform for hESC expansion and CM differentiation. It is capable of producing 190.5 ± 58.8 × 10⁶ HES-3 or 117 × 10⁶ hiPSC (IMR90) derived CMs per production run at a purity of 65.73 ± 10.73% and 46.65 ± 5.73% (cTnT) respectively, in a time frame of 19 days. This is equivalent to 31.75 ± 9.74 and 19.56 ± 0.44...
Comparing the platform to static MC cultures, the integrated platform demonstrates higher proliferative efficiency (18.70 ± 2.82 and 2.45 ± 0.12 cell expansion fold as compared to 7.20 ± 0.08 and 1.54 ± 0.12 during the expansion and differentiation phases respectively). This difference in cell yields can probably be attributed to diffusional limitation (especially oxygen) in static MC cultures (Kinney et al., 2011). The CM production capabilities of this platform (31.75 ± 9.74 CM/hESC seeded and 190.5 ± 58.8 × 10^6 CMs per production run) far exceed yields reported in literature for 3D MC and EB suspended cultures: Lecina et al. reported yields of 0.33 CM/hESC seeded in stirred spinner cultures using MCs (Lecina et al., 2010); Niebruegge et al. (2009) reported yields of 39.8 × 10^6 CMs per production run using an EB differentiation method in 125 mL stirred tank bioreactors; Ting et al. (2013) reported yields of 0.59 CM/hESC using a protocol in which pluripotent cells were expanded in MNL and differentiated as EBs. Current MNL differentiation protocols using costly growth factors are capable of producing 2 × 10^8 purified CMs per T225 flask (Burridge et al., 2012) which is three fold less in comparison to the production capabilities presented in this work (1.90 ± 0.58 × 10^8 per T-75 flask). Moreover, MNL platforms are not suitable for scale up due the limited surface area for cell growth, limited capabilities for monitoring and control, and requirement of extensive manual handling for multi-plate processes.

The number of CMs needed to regenerate an infarcted area after a heart attack is estimated to be from 1 × 10^8 to 2 × 10^9 (Jing et al., 2008). The integrated platform can be scaled up using larger agitators (e.g. Wave bioreactors) which are available at up to 500 L scale with full monitoring control and automatic handling (GE Life Sciences) and is capable of producing up to 1.33 × 10^12 (estimated based on current production yields). Moreover, volumetric productivity can be further optimized in these systems by increasing MC concentration and optimization of medium feed strategies as well as better monitoring of environmental conditions (e.g. pH and dissolved oxygen control as well as control of nutrients and waste products levels). Previous works have reported that the CM differentiation process can be affected by pH control, oxygen concentration (Niebruegge et al., 2009) and lactate concentration (Tohyama et al., 2013). The small molecule differentiation approach and the use of serum-free media also make the platform more cost effective and amenable to adaptation into GMP requirements.

During the hESC expansion phase, the observed difference in aggregate size and variance between the rocker (166 ± 88 × 10^5 μm^2) and spinner platforms (85 ± 42 × 10^5 μm^2) can be attributed to the difference in intensity of shear stress applied in the systems (Oncul et al., 2010). Previous flow characterization studies have shown that the minimum agitation speed to evenly suspend the aggregates in the spinner flask platform (25 rpm) generates at least 1.1 dyn/cm^2 (Santos et al., 2011; Sucosky et al., 2004). In comparison, flow characterization studies of rocking platforms for aggregates suspension show that shear forces of 0.1 dyn/cm^2 (Oncul et al., 2010) is required, a tenfold difference in comparison to the spinner platform. The aggregates generated in the integrated platform are uniform in size (148.94 ± 52.92 × 10^5 μm^2) and their generation does not require extensive cell handling that is required for EB generation (Kurosawa, 2007). Previous studies have shown that aggregate sizes and uniformity have an effect on CM differentiation efficiency with aggregate sizes between 200–800 μm (equivalent to 12.5 × 10^5–200.9 × 10^5 μm^2 assuming aggregates are spherical) considered to be the optimal for cardiomyogenesis (Niebruegge et al., 2009; Peerani et al., 2007). These values are in agreement with the size of aggregates obtained in rocker culture (148.94 ± 52.92 × 10^5 μm^2).

One of the major challenges in scaling up of hESC cultures is the effects of the mechanical stimulation generated by the hydrodynamic shear forces which is an inherent property of agitated cultures (Earls et al., 2013). Previous studies done with mouse embryonic stem cells have shown that fluid shear stress pre-conditioning promotes endothelial morphogenesis of cells within embryoid bodies (Nsiat et al., 2014). In addition, Metallo et al. (Peerani et al., 2007) reported that shear stress induces hESC elongation, up regulation of endothelial marker genes, and parallel alignment of hESC (Earls et al., 2013; Metallo et al., 2008). In this study, we found that the extent of the shear forces applied during the hESC expansion phase has an effect on CM differentiation. Specifically, cultures expanded in spinner cultures, which has a high rate of shear stress applied on the pluripotent cells during expansion, displayed 51.9% reduction in their capability to differentiate into CM (cTnT: 22.83 ± 2.56% %) in comparison to lower shear stress conditions in rocker cultures (cTnT: 47.50 ± 7.35%). However, a parallel study done in our group shows that by using different in-house types of MCs (smaller in size, more rigid and coated with purified extra cellular matrix molecules) and different agitation regime (static periods on day 1 and day 3 of differentiation), hESC cell propagated in spinner flasks can be efficiently used for differentiation to CMs (unpublished data). The reason for these effects is not clear and requires further studies to elucidate how hESC responds to mechanical stimuli and how this response affects CM differentiation capabilities.

We have found that the shift from pluripotent expanding cells to cells undergoing CM differentiation results in a parallel shift in cell growth properties: increase in doubling time (37 ± 7 h as compared to 131.46 ± 33.6 h) as well as, a change in glucose metabolism from anaerobic to aerobic (Y Lac/Glc = 1.99 ± 0.02 compared to 0.96 ± 0.08). Previous studies have also demonstrated a shift in energy metabolic phenotype of CMs as they undergo differentiation (Lopaschuk and Jaswal, 2010; Chung et al., 2007). Specifically, as CMs become terminally differentiated, the major source of energy changes from anaerobic glycolysis to the more efficient aerobic mitochondrial fatty acid beta-oxidation. Disrupting this respiratory change prevented mitochondrial organization and compromised the energetic infrastructure, causing deficient sarcomerogenesis and contractile malfunction (Chung et al., 2007).

The three culture platforms (static, intermittent agitated and continuously agitated) tested in this study present different oxygen/nutrient diffusion rates, oxygen saturation levels and shear force conditions. Specifically, the static platform has low nutrient/oxygen diffusion rates, low oxygen saturation levels and no shear force conditions. On the other extreme, the continuously agitated platform has high nutrient/oxygen diffusion rates, high oxygen saturation...
levels and high shear force conditions. The intermittent agitated platform follows a cyclic pattern of high and low levels of nutrient/oxygen diffusion rates, oxygen saturation levels and shear force conditions for the first 3 days and subsequently follows the same conditions as the continuously agitated platform. The results in this study indicate a low CM differentiation efficiency in continuously agitated cultures (cTnT < 5%) and an improved efficiency when comparing intermittent agitated vs. static cultures (65.73 ± 10.73% vs 47.50 ± 7.35% cTnT positive cells). It is important to note that the increase in oxygen saturation levels and CM differentiation efficiencies in the intermittent agitated platform compared to the static platform is contradictory to previous works showing an increase in CM differentiation efficiency in low oxygen tension environments (Burr ridge et al., 2011; Niebruegge et al., 2009). As such, we suggest that low shear forces play a more important role in an agitated environment for CM differentiation in comparison to oxygen saturation levels. Multiple protocols initiate CM differentiation through activation of the TGF-β pathway directly by addition of growth factors (Activin A and BMP4) (Burr ridge et al., 2011; Kattman et al., 2011; Yang et al., 2008; Willems et al., 2011) or indirectly with the addition of small molecules (CHIR or BIO) (Lian et al., 2012). In addition, the temporal exposure of these growth factors and small molecules has been found to be extremely critical to differentiation process (Kattman et al., 2011). Thus, we have tried to correlate the inhibitory effect of agitation on CM differentiation and the expression levels of SMAD7, an inhibitor of the TGF-β pathway which was previously reported to be upregulated during increased shear stress (Wang et al., 2008). Interestingly, we found that SMAD7 is expressed at higher levels in differentiating HES-3 cells maintained at continuous agitation for the first 3 days as compared to control cultures maintained by intermittent agitation (Supplementary Fig. 1A). Thus, it can be hypothesized that the inhibition of the TGF-β pathway as a result of increased expression levels of SMAD7 due to shear stress in agitated cultures, negatively affects the CM differentiation process. However, the elucidation of this mechanism will require further extensive research that will be done in the future.

In conclusion, a 3D suspended MC rocker platform, similar to a wave bioreactor, that integrates both hESC expansion and CM differentiation has been developed and shown to be capable of producing the highest quantities of CMs to date (190.5 ± 58.8 × 10^6 cTnT positive CM/production run; 31.75 ± 9.74 cTnT positive CM/hESC). CMs expressed cardiac-specific transcriptional factors, structural and functional genes, and generation of cross-striated muscle structure that recapitulate the process of cardiomyogenesis. The platform requires minimal human-intervention, lab space foot print and utilizes a cost effective and defined structure that recapitulate the process of cardiomyogenesis.

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Appendix A. Supplementary data

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References


