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

Sherwin Ting[a], Alan Lam[a], Gerine Tong[a], Allen Chen[a], Heming Wei[b], JianJun Wu[b], Yue Ning Lam[a], Shaul Reuveny[a], Steve Oh[a,*]

[a] Bioprocessing Technology Institute, A*STAR (Agency for Science, Technology and Research), Singapore 138668, Singapore
[b] National Heart Centre Singapore, Singapore 169609, Singapore

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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

* Corresponding author at: Bioprocessing Technology Institute, 20 Biopolis Way #06-01 Centros, Singapore 138668, Singapore.
E-mail address: steve_oh@bti.a-star.edu.sg (S. Ting).

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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

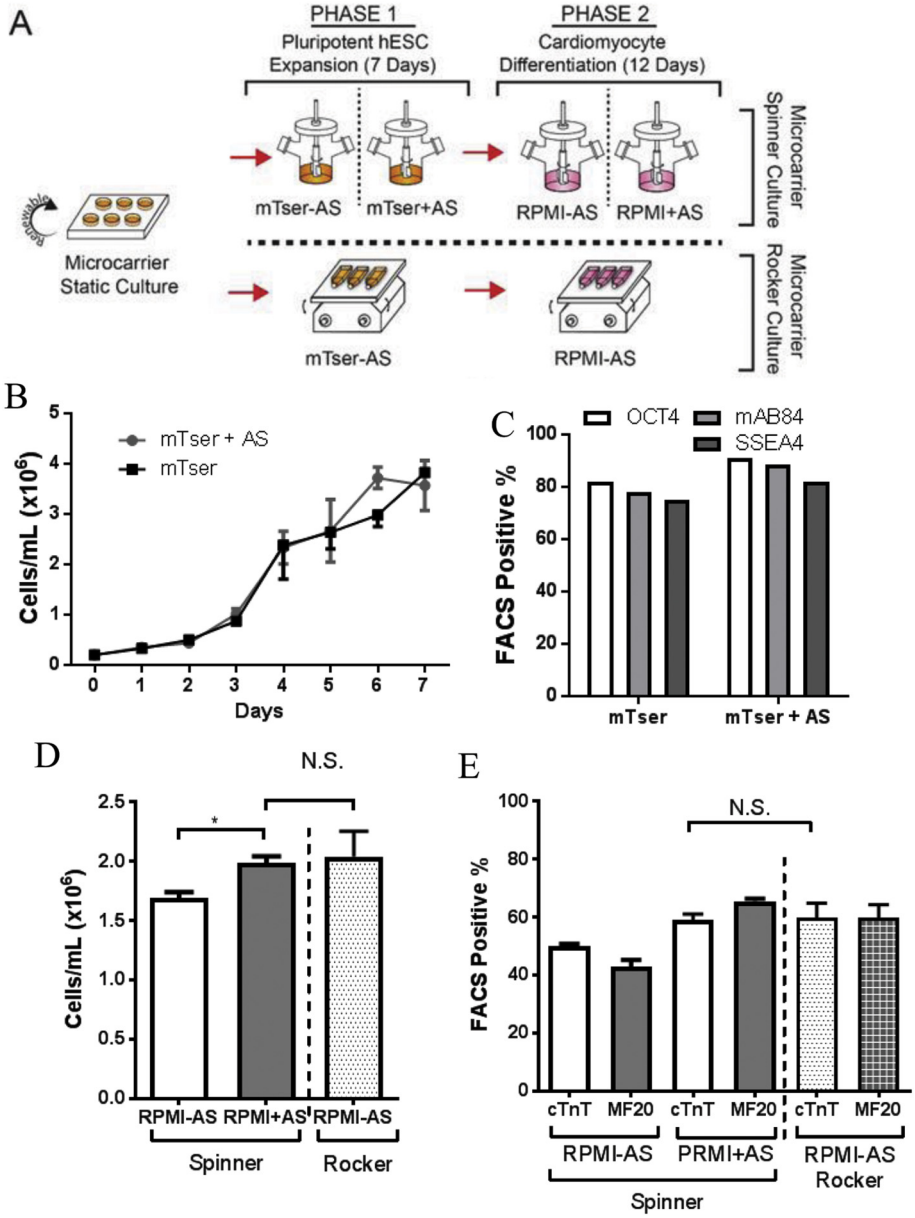


Fig. 1. Effect of Ascorbic acid on HES-3 cell expansion and CM differentiation efficiency.
HES-3 cells were expanded (phase 1) and differentiated (phase 2) in MC culture on spinner cultures and rocker (wave-type) platforms. Spinner cultures were agitated intermittently (66/6) at 25–30 rpm and rocker culture rocked at 30 oscillations/min at a tilt angle of 12° during the first 3 days of differentiation. A schematic diagram of spinner culture platform to determine the effect of AS on cell expansion and differentiation is shown in (A), with rocker cultures without ascorbic acid as the control. The addition of 64 mg/mL of AS to the spinner culture during cell expansion did not have a significant effect on cell growth (B) and pluripotency (C). However, addition of 64 mg/mL of AS at the differentiation stage results in an increase in cell yield (D) and CM yield (E) which was comparable to that found in the control rocker platform. ($n = 3$, *p < .01, Bonferroni corrected).

refreshing 80% of the original medium volume with differentiation medium and seeded at a concentration of 1×10^6 cells/mL into (I) ULA T-25 flask containing 30–33 mL medium on a rocker shaker (MC rocker culture) at rocking rate of 30 oscillations/min at a tilt angle of 12° or (II) 125 mL disposable spinner flasks containing 40–100 mL medium at 25–30 rpm respectively. Differentiation media consists of RPMI 1640 medium supplemented with B27-insulin (ThermoFisher Scientific) and 213 μg/mL of L-ascorbic acid 2-phosphate (Sigma-Aldrich). Differentiation was done according to a protocol previously described (Lian et al., 2013). Briefly, CHIR99021 (CHIR) (Selleckchem) in differentiation medium was added at a concentration of 12 μM for the first 24 h and then removed *via* medium change. On day 3 of differentiation, cells were treated with 5 μM IWR-1 (Selleckchem) in differentiation medium. IWR-1 was removed *via* a medium change on day 5 and cells were maintained in differentiation medium thereafter. Different agitation regimes ((I) 66/6, (II) 8/1, (III) continuous agitation) were introduced on the first 3 days of differentiation to optimize and evaluate the effects of shear stress on cardio-genesis. Cultures were harvested for cell count and FACS analysis between days 10 to 12.

3. Purification of CMs in MC spinner flask cultures (phase 3)

Differentiated CM/MC aggregates obtained from MC spinner cultures in phase 2 were maintained in the same vessel for the purification step. Aggregates were washed once by refreshing 80% of the original medium volume with purification medium. The cultures were agitated for 5–10 days at 25–30 rpm (37 °C with 5% CO_2) with 80% medium change every other day. Purification media consists of RPMI 1640 medium without D-glucose (ThermoFisher Scientific), 5 mM of Sodium L-Lactate (Sigma-Aldrich), and 213 μg/mL of L-ascorbic acid 2-phosphate (Sigma-Aldrich).

3.1. Immunohistology

CM cell aggregates were harvested on day 5 of the purification step (Phase 3), washed and fixed with Fixation Medium (Medium A) (ThermoFisher Scientific). Cryo-sectioned slides were then prepared. Afterward, the slide samples were permeabilized and blocked by 0.1% Triton X-100 (Sigma-Aldrich) and 10% goat serum (Dako) respectively.

The following antibodies were used: anti-MYL-2A (MLC-2A; Synaptic Systems) and anti-troponin-T (ThermoFisher Scientific). Slow Fade Glow with DAPI (4′6-diamidino-2-phenylindole) (ThermoFisher Scientific) was used for nuclear staining. The fluorescence was observed using a Nikon TI-E fluorescence microscope coupled with Nikon imaging software, NIS elements.

3.2. Flow cytometry

Aggregates were dissociated into single cells with TrypLE™ Express (ThermoFisher Scientific), the cell/MC suspension was filtered through a 40-μm cell strainer (Becton Dickinson) to remove the MCs. Subsequently, cells were fixed and permeabilized with FIX & PERM® Cell Permeabilization Kit (ThermoFisher Scientific) according to manufacturer's directions. The following primary antibodies were used: anti-myosin heavy chain (anti-MHC; MF20, dilution 1:200; Developmental Studies Hybridoma Bank) and anti-troponin-T (anti-cTnT; 1:200; ThermoFisher Scientific). Alexa Fluor 647® goat anti-mouse (ThermoFisher Scientific) 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).

3.3. qRT-PCR

Total RNA was isolated from the cells ($> 5 \times 10^6$) obtained during the first 3 days of differentiation, using Trizol (Invitrogen) and RNeasy Mini Kit (Qiagen) following the supplier's protocol. One μg of total RNA was used for reverse transcription using Maxima First Strand cDNA synthesis kit (Fermentas). Real-time PCR was performed on an ABI 7500 system (Applied Biosystem) by applying a standard two-step amplification protocol to detect mRNA expression. Relative expression values were obtained by normalizing Cτ values of the tested genes to the Cτ values of the housekeeping gene GAPDH using the ΔΔCτ method. Final results for gene expression were presented as $2^{-\Delta\Delta C\tau}$. The following genes were tested: Nanog, LIN28, Mesoderm posterior 1 (Mesp-1), β-Catenin, E-Cadherin, T cell factor-1 (TCF-1), Runt-related transcription factor 1 (RUNX1), Mesogenin 1 (MSGN1), T-Box 6 (TBX6), and Hypoxia-inducible factor 1-alpha (HIF1α). Primer sequences are provided in Supplementary Table 1.

3.4. Metabolite analysis

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

3.5. Patch-clamp

Action potentials (AP) were measured by the patch-clamp technique under whole-cell configuration using isolated single cells as described previously (Wu et al., 2017). The signal was amplified using an axon 700B patch clamp amplifier (Axon Instrument, Sunnyvale, USA) and low-pass filtered at 5 kHz. Patch pipettes were fabricated from glass capillaries (O.D, 1.5 mm; I.D, 0.9 mm) using a Sutter P-97 microelectrode puller (Novato, CA, USA) and the tips were heat polished with a microforge (NARISHIGE MF-900) to gain a resistance of 2–4 MΩ. The electrical signals were sampled at 2.5–10 kHz and filtered at 2 kHz using a low-pass filter. Data acquisition was performed using the Digidata 1440A (axon instrument). Data analysis and fit were carried out using clamp fit 10.2 (axon instrument) and Origin 7.0 software (Origin Lab Corporation). pClamp software (Version8.1; Axcon Instrument) was used to generate voltage-pulse protocols, acquire and analyze data. APs were recorded under current-clamp mode at 35 °C.

Pipette solution contained (in mM): KCl 130, NaCl 5, $MgCl_2$ 1, MgATP 3, EGTA 10, and HEPES 10, with pH adjusted to 7.2 with KOH. Extracellular solution (Tyrode's solution) contained (in mM) NaCl 140, KCl 5.4, CaCl2 1.8, MgCl2 1, glucose 10, HEPES 10, with pH adjusted to 7.4 with NaOH. The AP parameters analysed were AP amplitude (APA), overshoot, maximal diastolic potential (MDP), AP durations (APD) at 50% and 90% of repolarization (APD50 and APD90), the maximum upstroke velocity or maximum rate of depolarization (dV/dt_{Max}) and beating frequency or heart rate (HR).

The electrophysiological data from independently differentiated batches were pooled for analyses as described previously (Liu and Backx, 2014). The recorded APs were used to classify the hPSC-derived cardiomyocytes into atrial-like and ventricular-like cardiomyocytes subtypes.

3.6. Cell count

Total and viable cell concentration was determined by the nuclei count method with DAPI using NucleoCounter NC-3000 (Chemometec) according to the manufacturer's instructions.

3.7. 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 as previously described (Ting et al., 2014).

3.8. 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 (SD) of at least 3 repetitions.

4. Results

Previously, we have reported efficient production of CMs (yield of $1.41 \pm 0.28 \times 10^6$ CM/mL and purity of 56.19% ± 13.49%) using a wave type (a side to side rocker) reactor (Ting et al., 2014). However, attempts to scale up the system resulted in lower and variable yields (data not shown), this can be explained by the increase in shear and variability of flow velocity as the scale of wave-type systems increase (Kalmbach et al., 2011). As such, there was a need to adapt and optimize the platform to a more linearly scalable conventional stirred system capable of producing high CM yields.

5. Optimizing CM production in stirred spinner flasks

Our previous publication demonstrated that continuous agitation of wave-type rocker reactor during the first 3 days of differentiation is the major reason for low differentiation efficiency and applying intermittent agitation (66/6) resulted in an increase in CM yields (Ting et al., 2014). For this reason, we adopted this method to differentiate HES-3 cells in spinner culture, which is intermittent agitation of 66/6 during the first 3 days of differentiation (which resulted in CM yields of $1.70 \pm 0.06 \times 10^6$ cells/ml and CM purity of 50.00 ± 1.31%; (Fig. 1 D & E).

In order to further increase CM yields, we tested the addition of AS to the expansion and differentiation medium and carried out further optimization of the agitation regime.

5.1. Addition of ascorbic acid (AS)

To test the effect of the addition of AS into culture media on CM differentiation efficiency, we added 64 mg/mL AS into the expansion and differentiation medium in the spinner cultures in comparison to non-AS supplemented cultures. Non-AS supplemented wave-type rocker platforms were used as a control. All cultures were agitated with 66/6 intermittent

agitation regime during the first 3 h of differentiation (Fig. 1A).

Results presented in Fig. 1B & C demonstrate that addition of AS to the spinner cultures did not affect significantly the cell growth and pluripotency during the expansion phase, although AS addition resulted in a slight increase in the growth rate of the cells at day 6 as compared to the culture without AS. However, during the differentiation phase, from Fig. 1D & E, we observed that the addition of AS to the spinner culture results in increase in cell yield (from $1.70 \pm 0.06 \times 10^6$ to $1.99 \pm 0.11 \times 10^6$ cells/mL; $p > .05$) and CM purity (from 50.00 ± 1.31% to 59.10 ± 2.82% for cTnT positive cells; $p = .05$). This equates to a total increase of 38.42% in CM yield ($0.84 \pm 0.03 \times 10^6$ CM/mL *vs* $1.17 \pm 0.07 \times 10^6$ CM/mL for cultures without AS and with AS respectively). Differentiation efficiencies for spinner cultures with AS added were similar to that obtained in the rocker cultures ($1.17 \pm 0.07 \times 10^6$ CM/mL *vs* $1.22 \pm 0.40 \times 10^6$ CM/mL).

5.2. Optimizing agitation regime

In order to understand the effect of agitation on CM differentiation efficiency, we compared HES-3 agitated in spinner culture either continuously at a rate of 25–30 rpm or intermittently (66/6) during the first 3 days of differentiation (Fig. 2). Similar to results obtained previously

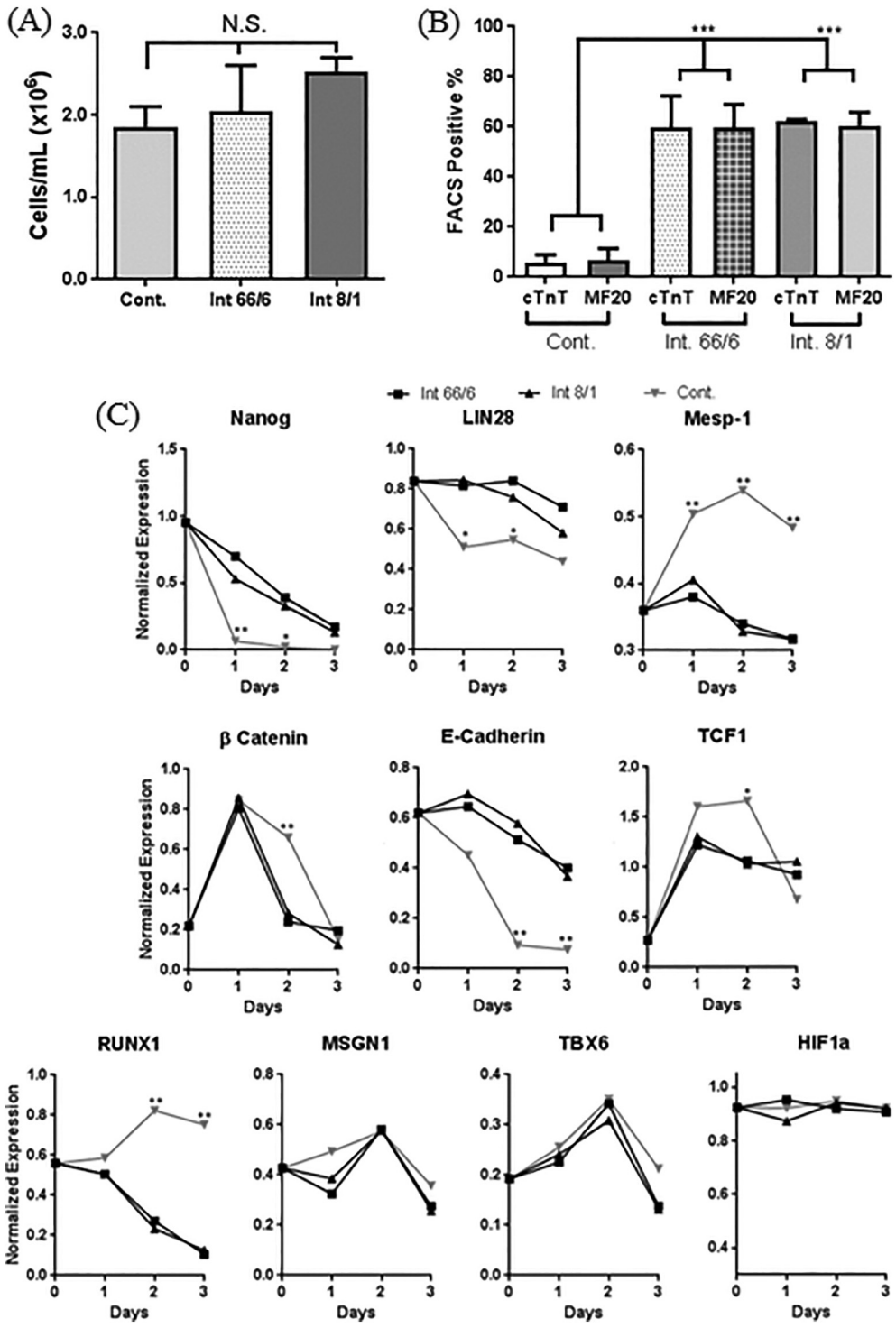


Fig. 2. Effect of shear stress on WNT modulation during the CM differentiation process in stirred spinner flask.
HES-3 cell-MC aggregates obtained from 7 days old stirred spinner culture were differentiated for 12 days in the same spinner flasks. The cultures were either maintained at continuous agitation (Cont.; 30 rpm) or with intermittent agitation (Int. 66/6.; 66 min off 6 min on and Int. 8/1.; 8 min off 1 min on) during the first 3 days of differentiation. The continuous agitation regime did not have significant effects on the cell yield (A) however it resulted in drastic decrease in CM yields (B). Expression of pluripotent (Nanog and LIN28), mesodermal (Mesp-1), WNT modulation gene (β-Catenin, E-Cadherin and TCF-1), early haematopoietic marker (RUNX1), paraxial mesoderm markers (MSGN1 and TBX6), and hypoxia-inducible factor (HIF1α) were measured by qRT-PCR analysis during the first 3 days of differentiation shows that continuous agitation resulted in faster decrease in Nanog and LIN28, leading to a sharp increase in the expression of Mesp-1 followed with the increase of the expression of β-Catenin and TCF-1 with a drastic decrease in E-Cadherin on day 1 of differentiation. There is also an upregulation of RUNX1 expression in continuous agitation, as compared with intermittently agitated cultures, showing that that the differentiation is not directed towards cardiac lineage but rather to haematopoietic progenitors. Moreover, changing the agitation regimes from 66/6 to 8/1 did not affect the level of expression of the tested genes (C). ($n = 3$, $^*p < .01$, $^{**}p < .05$ and $^{***}p < .0001$, Bonferroni corrected).

in the wave-type reactor on rocker platform[1], continuous agitation in spinner flask did not affect cell yield (Fig. 2A); however, it utterly inhibits the cardiomyocyte differentiation efficiency (only 5.87% cTnT-positive cells; Fig. 2B). By applying 66/6 intermittent agitation during the first 3 days of differentiation, a significant increase in CM purity (59.63% cTnT-positive cells *vs* 5.87% cTnT-positive cells (Fig. 2B) in the intermittent and continuous cultures respectively) was observed.

Real-time PCR analysis (Fig. 2C) showed that there was a significant difference in the expression of pluripotency markers (Nanog and LIN28), mesoderm germ layer marker (Mesp-1) and WNT signaling factors (β-Catenin, E-Cadherin and TCF1) when comparing continuous and intermittent stirred cultures. Continuous agitation resulted in a faster decrease in Nanog and LIN28, leading to a sharp increase in the expression of Mesp-1 on day 1 of differentiation. This is followed by an increase of the expression of β-Catenin and TCF-1 with a drastic decrease in E-Cadherin, implying the Wnt pathway is being activated on day 1 and continued on day 2. On the other hand, intermittently agitated cultures resulted in a gradual decrease in Nanog but no change in LIN28 expression, leading to a small increase in the expression of Mesp-1 on day 1 of differentiation and this expression was subsequently decreased afterward. The Wnt signaling was also activated as illustrated by the increase in the expression of β-Catenin and TCF-1 on day 1, and followed by a decrease in E-Cadherin expression. However, unlike continuous agitation, the level of β-Catenin was drastically decreased and returned to basal levels on day 2, implying that the Wnt pathway is being repressed. The gene expression of other non-cardiac lineages (RUNX1, MSGN1, TBX6) shows that RUNX1 (an early haematopoietic cells marker) is highly expressed on day 2 only in continuously agitated culture suggesting that these cultures tend to differentiate into blood cell lineage. Whereas, the expression of MSGN1 and TBX6 (paraxial presomitic mesoderm markers), as well as HIF1α (a master transcriptional regulator of the adaptive response to hypoxia) were similar in continuous or intermittent agitation cultures. Moreover changing of the intermittent agitation regime from 66/6 to 8/1 had no significant effect on gene expression.

We further explored the effect of the long stationary stage (66 min) of the intermittent agitation regime on oxygen supply to the culture (Fig. 3A). During this stage, the dissolved oxygen concentration in the spinner flask is reduced at a rate of 3.5% of air saturation/min, achieving anoxic conditions at about 10 min (< 20% air saturation). This means that during the intermittent agitation regime of 66/6, the culture is maintained in anoxic conditions for about an hour. Thus we have modified the intermittent agitation regime by shortening the static interval to 8 min so that DO is maintained about 30%–40% air saturation, followed by 1 min agitation to bring the culture back to 100% air saturation.

To this end, a study on the effect of this new intermittent agitation regime (8/1) on CM differentiation was carried out. hESC-MC culture expanded in 125 mL disposable spinner flasks (phase 1) were further differentiated to CMs (phase 2) in the same vessel under the following agitation conditions: (I) Continuous agitation (II) Intermittent agitation (8/1) and (III) Intermittent agitation (66/6). As expected, in continuous agitated cultures, cells expanded to a higher level ($2.99 \pm 0.05 \times 10^6$ cells/mL) however they showed a significant decrease in both cTnT and MF20 positive cells

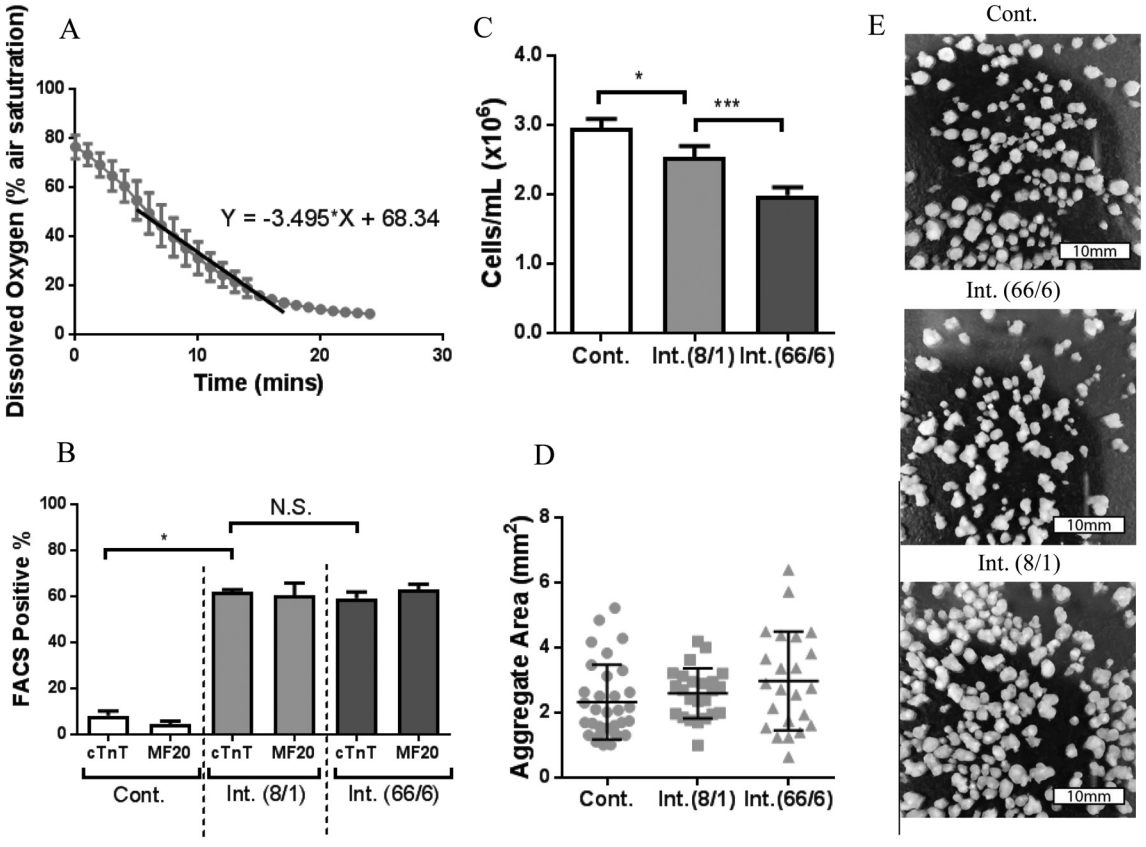


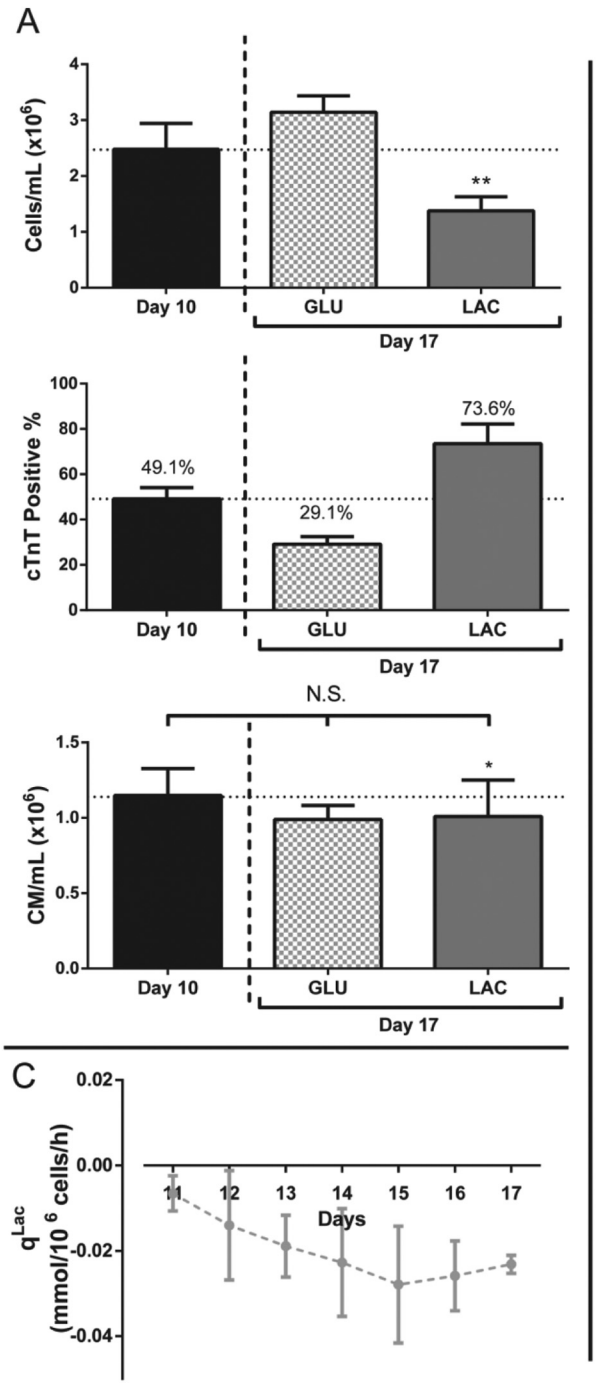
Fig. 3. Optimizing intermittent agitation regime in spinner flask cultures during the first 3 days of cardiomyocyte differentiation.
HES-3 cell-MC aggregates obtained from 7 days old expanded spinner culture were diluted to a cell density of 1×10^6 cells/mL with a total volume of 50 mL to undergo a CM differentiation process maintained at 2 intermittent agitation regimes (Int. 8/1 or Int. 66/6) during the first 3 days of differentiation compared to a continuously agitated culture (Cont.). Cultures maintained under non-stirred static condition for 30 min demonstrated fast decrease in dissolved oxygen (DO) levels (A). CM purity (B), cell yields (C), the distributions of aggregate size (D) and microscopic images of the aggregates (E) obtained under the different agitation conditions showed that 8/1 agitation regime resulted in similar CM purity (B) but higher cell yield (C) and more uniform aggregate size distribution (D, E). (n = 3, *p < .01 and ***p < .0001, Bonferroni corrected).

(8.2 ± 3.4% and 4.5 ± 2.6%, respectively) compared to both intermittently stirred cultures (Fig. 3B). The two agitation regime achieved similar CMs purity (about 60% after 12 days of differentiation) (Fig. 3B), however the new 8/1 intermittent stirring regime improved the cells densities obtained from 2.04 ± 0.44 × 10^6 to 2.53 ± 0.06 × 10^6 cells/mL, presumably due to better oxygen transfer (Fig. 3C). Furthermore, aggregates sizes obtained in the new intermittent stirring regime (8/1) cultures were more homogenous in size (about 2–4 mm^2; Fig. 3D & E) probably as a result of the elimination of cell aggregation occurring in the long static phase (66 min). Overall, the optimized intermittent agitation regime (8/1) produced 1.50 ± 0.10 × 10^6 CM/mL, a 22% increase in CM production efficiency in comparison to cultures with an intermittent agitation regime of (66/6) which produced 1.23 ± 0.07 × 10^6 CM/mL.

5.3. Incorporation of additional lactate based CM purification step into the spinner based CM differentiation process

CM purity is an important key parameter in CM production. Unwanted cell populations generated during CM differentiation may be of concern for future applications. Recently, it has been reported that CMs are able to survive in glucose-depleted conditions, as they are able to efficiently use lactate to fuel the TCA cycle (Burridge et al., 2014; Lian et al., 2012). This is contrary to most cell types, especially hPSCs, allowing a "purification effect" to be established (Burridge et al., 2014). As such, we investigated integrating this step into our spinner based scalable CM bioprocess platform.

Results presented in Fig. 4 showed that HES-3 cells expanded (phase 1) for 7 days and differentiated to CMs (phase 2) in 125 mL disposable



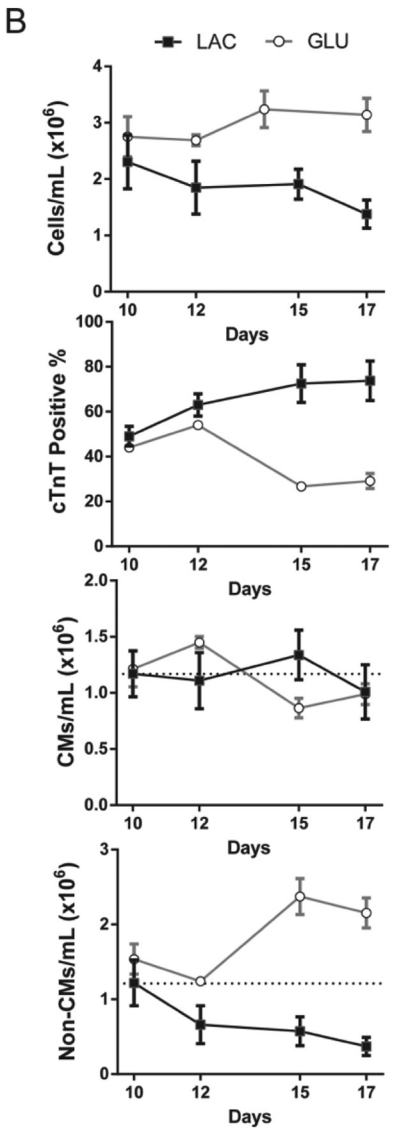


Fig. 4. Effect of addition of lactate purification step to MC based spinner culture expansion and differentiation process on cell growth and CM purity.
10 days old CM differentiated MC spinner cultures were treated for an additional 7 days with lactate based medium (5 mM Lactate in RPMI without glucose) and glucose based medium (RPMI with glucose). Total cell count, CM yield and CM purity were determined after 7 days of lactate purification (A). Kinetics of total cells, CM purity, CM cell growth and non-CM cell growth in lactate and glucose media were monitored (B) and specific consumption rate of lactate in the CM purification media during the 7 days was calculated (C). (n = 3, **p < .001, Bonferroni corrected).

Table 1
Effects of glucose and lactate addition during purification process on cell metabolism and CM purity and yields.

Concentration		Cell count	CM purity		CM yield	Specific consumption rates	
Lactate (mM)	Glucose (mg)	Cells (cells/mL × 10^6)	cTnT (%)	MF20 (%)	CM/mL	Glucose (mmol/10^6 cell/h)	Lactate (mmol/10^6 cell/h)
0	0	0.95 ± 0.18	71.7	80.8	0.67 ± 0.06	Na	Na
5 mM	0	1.92 ± 0.27	72.5	75.6	1.34 ± 0.26	Na	(−0.019)
5 mM	200 mg	2.13 ± 0.28	29.9	29.0	0.64 ± 0.04	0.028	0.017
5 mM	1000 mg	2.92 ± 0.16	26.3	26.2	0.76 ± 0.04	0.106	0.220
5 mM	2000 mg	3.06 ± 0.21	19.6	18.0	0.60 ± 0.04	0.136	0.145

spinner flasks for 10 days can be further purified by exposing the cells to purification media RPMI +5 mM Lactate without glucose (LAC). This was done in comparison to control culture maintained in RPMI with glucose (GLU). The lactate treatment led to a decline in total cell number (from 2.48 ± 0.46 × 10^6 cells/mL to 1.38 ± 0.25 × 10^6 cells/mL) and increase in CM purity (from 49.09 ± 5.04% to 73.55 ± 8.89% of cTnT-positive cells; Fig. 4A). In total, during the 7 days process, we saw an increase in CM purity without a significant change in concentration of CM (1.0 × 10^6 CM/mL) (Fig. 4A). However, in control cultures which were maintained in glucose medium (GLU), we saw an opposing phenomenon of an increase in cell numbers (from 2.48 ± 0.46 × 10^6 cells/mL to 3.14 ± 0.29 × 10^6 cells/mL) with a decrease in CM purity (from 49.09 ± 5.04% to 29.10 ± 3.39% of cTnT-positive cells; Fig. 4A).

In order to further understand the purification process, we monitored cell growth daily. Results presented in Fig. 4B shows that during the 7 days purification process in lactate medium, the concentrations of CMs do not change while there is a drastic decrease (~76%) in the concentration of the non-CMs concentration. In the glucose medium, the opposite happened, as a drastic increase in non-CM cells was noted (~40% increase) with the maintenance of CM concentration.

It is important to note that during the lactate based purification process, lactate was consumed as the carbon source at a rate of 0.019 mmol/10^6 cells/h (Fig. 4C, Table 1) and is probably consumed primarily by CMs; while in the glucose medium, glucose is consumed and lactate is produced (Table 1), leading probably to the growth of non-CM cells. The presence of even small amounts glucose (200 mg/L) in the lactate "purification medium" prevents the CM purification process (Table 1). Interestingly, even in the absence of both lactate and glucose, high CM purity (~70% cTnT) was achieved but with lower cell and CM yield (< 1 × 10^6 cells/mL) at the end of the purification process (Table 1). We can assume that the effect of purification is achieved by the absence of glucose causing cell death of non-CMs while lactate is used as carbon source for maintaining CM metabolism.

Next, we determined the optimal timing to initiate CM purification, in which three-time points were selected - Day 7, Day 10 or Day 12 post-differentiation (Fig. 5). These timings were selected based on previous publications. Specifically, Day 7 was chosen based on the study by Lian et al. with the emergence of cardiac progenitors differentiated from hPSC (Lian et al., 2012). Day 10 was chosen as it was used as the start timing in a similar purification protocol developed by Burridge et al. (2014). Day 12 was considered to determine if a later exposure to purification would produce higher CM yields.

Results presented in Fig. 5A shows that the optimal timing for initiating the purification process, for a day 17 harvest, is at Day 10 of the

A

Lactate Purification Timing (Starting Day)	CM Yield (CM/mL)	Timing (Day) (Day)	Total Cell Count (x10^6 cells/mL)	CM Purity cTnT (%)	MF20 (%)	Agg Size (mm^2)
Day 7	1.06±0.16	Day 10	2.06±0.36	56.32±0.02	66.26±0.13	1.45±0.68
Day 10	1.34±0.25	Day 15	1.91±0.27	72.50±8.34	75.63±10.61	1.95±0.80
Day 12	1.47± 0.31	Day 17	2.00±0.15	73.45±18.31	77.13±10.21	1.65±0.82

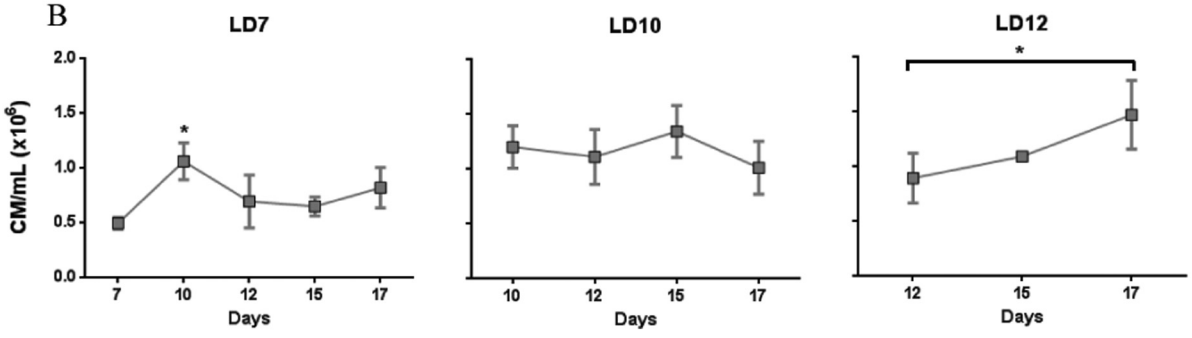


Fig. 5. The optimal timing for initiating and duration of lactate purification process.
CM purification was started by changing differentiation medium into lactate purification medium on Day 7 (LD7), Day 10 (LD10) or Day 12 (LD12). Total cell count, CM yield, CM purity and aggregate size were measured (A). Growth kinetic profiles for each purification culture were measured to determine the optimal duration of the process (B). (n = 3, *p < .01 and **p < .001, Bonferroni corrected).

differentiated cultures (1.34 ± 0.25 × 10^6 CM/mL and 72.50 ± 8.34% cTnT). Earlier introduction of lactate purification on Day 7 resulted in lower cell yield and CM purity (only 1.06 ± 0.16 × 10^6 CM/mL and 56.32 ± 0.02%), while postponing the introduction to Day 12 did not significantly improve cell yield and purity (1.47 ± 0.31 × 10^6 CM/mL and 73.45 ± 18.31% cTnT; results similar to Day 10). Detailed cell growth kinetics after the initiation of purification is shown in Fig. 5B. The results demonstrated that the purification process should not go beyond 5 days for the cultures where the lactate purification was started on Day 10. A maximum CM yield was achieved after 5 days post-treatment of lactate (day 15; 1.34 ± 0.24 × 10^6 CM/mL), whereas a significant drop was observed, 7 days post-treatment of lactate (day 17, 1.01 ± 0.24 × 10^6 CM/mL). Therefore, the optimal protocol for CM purification in spinner cultures is to start the process on day 10 of post-differentiation for 5 days.

5.4. Integrate PSC expansion, differentiation and purification process in stirred MC spinner cultures

The results obtained above were incorporated into an integrated one unit expansion (7 days) differentiation (10 days) and purification (5 days) process. Several production runs were done in order to explore process reproducibility, and on additional iPSC line (IMR-90) was differentiated using the same process in order to demonstrate universality. Cell growth, CM yields, during expansion, differentiation, and purification are summarised in Table 2.

During hESC expansion, cell yields of 3.50 ± 0.51 × 10^6 cells/mL (HES-3; n = 3) and 3.01 ± 0.65 × 10^6 cells/mL (IMR-90; n = 3) with viability > 90% and pluripotent markers > 80% were achieved (Table 2A).

After the differentiation phase, there was an overall 2.31 ± 0.47 fold expansion with a cell density of 2.31 ± 0.47 × 10^6 cells/mL achieved in HES-3 differentiation (n = 17; viability above 90%). Expression levels of cTnT and MF20 markers were 48.11 ± 4.02% and 51.75 ± 10.28%, respectively (Table 2B). Altogether, a CM density of 1.11 ± 0.19 × 10^6 CM/mL was achieved after the 10-day differentiation process of HES-3. Similarly, there was an overall 1.97 ± 0.43 fold expansion with a cell density of 1.97 ± 0.43 × 10^6 cells/mL in IMR-90 differentiation (n = 17). Expression levels of cTnT and MF20 markers were 61.75 ± 18.61% and 67.40 ± 15.22%, respectively, with overall CM density of 1.22 ± 0.38 × 10^6 CM/mL achieved.

CM/MC aggregates from the differentiation process were directly transferred to the purification phase for 5 days. In differentiated HES-3, a cell density of 1.91 ± 0.26 × 10^6 cells/mL with expression levels of 72.50 ± 8.35% and 74.94 ± 12.08% for cTnT and MF20 respectively was achieved (n = 15). In differentiated IMR90 cultures, a cell density of 1.55 ± 0.0.43 × 10^6 cells/mL with expression levels of 83.12 ± 8.73% and 84.08 ± 12.30% for cTnT and MF20 respectively was achieved (n = 15). The significant drop in cell density was expected as the purification media did not contain any glucose to sustain the survival of non-CM cells. Despite a lower cell count, there was an increase in the overall CM yield with 1.38 ± 0.22 × 10^6 CM/mL (HES-3 culture) and 1.29 ± 0.42 × 10^6 CM/mL (IMR90 culture) after 5 days of purification.

The entire integrated process which took 22 days (7 days hESC expansion, 10 days differentiation and, 5 days purification) resulted in 242.3 ± 49.6 × 10^6 CMs/production run that started with an initial HES-3 volume of 50 mL. Likewise, a total of 201.7 ± 75.7 × 10^6 cTnT positive CMs per production run was produced with a purity of 83.12 ± 8.73% that started with an initial of 50 mL of IMR-90 volume.

5.5. Characterization of the CMs obtained from the integrated expansion, differentiation and purification process

Fig. 6A shows the immunofluorescence CM structure of the cell/MC aggregates after purification demonstrating positive staining for cardiac troponin (cTnT) and myosin light chain (MYL-2A) and displaying typical cross-striations of sarcomere structures.

Electrophysiological properties of the CM pre- (Day 10) and post- (Day 15) purification analysed by whole-cell patch clamp recordings (Fig. 6B) demonstrate similar Ventricular-like, Atrial-like, and Nodal-like activity. Cells exhibiting ventricular-like AP characteristics were predominant in the CM population, with about 80.0% of total cells (Fig. 6C). CMs derived from cultures pre- and post-purification also showed no signs of maturation with no significant changes in maximum diastolic potential (MDP), action potential amplitude (APA) and maximal rate of depolarization (dV/dt_{max}) for both ventricular-like and atrial-like CMs (Fig. 6D). Moreover, AP duration at 50% and 90% of repolarization (APD50 and APD90) were found to be rather high in ventricular-like CMs but with no obvious difference between pre- and post-purification CMs (Fig. 6D). Beating rates (HR) also appeared similar in both experimental groups (Fig. 6D). Together, the results suggest that the purification phase does not alter CM characteristic and phenotype.

Table 2
Integrated one unit expansion, CM differentiation and purification process of hESC (HES-3) and iPSC (IMR-90).

Culture Condition (Day 7)	Cell Expansion		Pluripotent Markers		
	Viable Cell Count (cells/mL)	Cell Expansion Fold	OCT (%)	mAB84 (%)	SSEA4 (%)
(A) hESC Expansion phase					
HES-3	3.5 ± 0.51 ×10^6	17.51 ± 2.55	86.27 ± 4.43	83.20 ± 7.21	89.30 ± 10.32
hiPS (IMR-90)	3.01 ± 0.65 ×10^6	15.15 ± 0.32	94.25 ± 1.06	93.09 ± 2.62	93.20 ± 4.52

Culture Condition (Day 10)	Cell Expansion		Cardiomyocyte FACS markers		Production
	Viable Cell Count (cells/mL)	Cell Expansion Fold	cTnT (%)	MF20 (%)	Cardiomyocytes (CM/mL)
(B) Cardiomyocyte Differentiation Phase					
HES-3	2.31 ± 0.47 ×10^6	2.31 ± 0.47	48.11 ± 4.02	51.75 ± 10.28	1.11 ± 0.19 ×10^6
hiPS (IMR-90)	1.97 ± 0.43 ×10^6	1.97 ± 0.43	61.75 ± 18.61	67.40 ± 15.22	1.22 ± 0.38 ×10^6

Culture Condition (Day 5)	Viable Cell Count (cells/mL)	Cardiomyocyte FACS markers		Production
		cTnT (%)	MF20 (%)	Cardiomyocytes (CM/mL)
(C) Cardiomyocyte Purification Phase				
HES-3	1.91 ± 0.26 ×10^6	72.50 ± 8.35	74.94 ± 12.08	1.38 ± 0.22 ×10^6
hiPS (IMR-90)	1.55 ± 0.43 ×10^6	83.12 ± 8.73	84.08 ± 12.30	1.29 ± 0.42 ×10^6

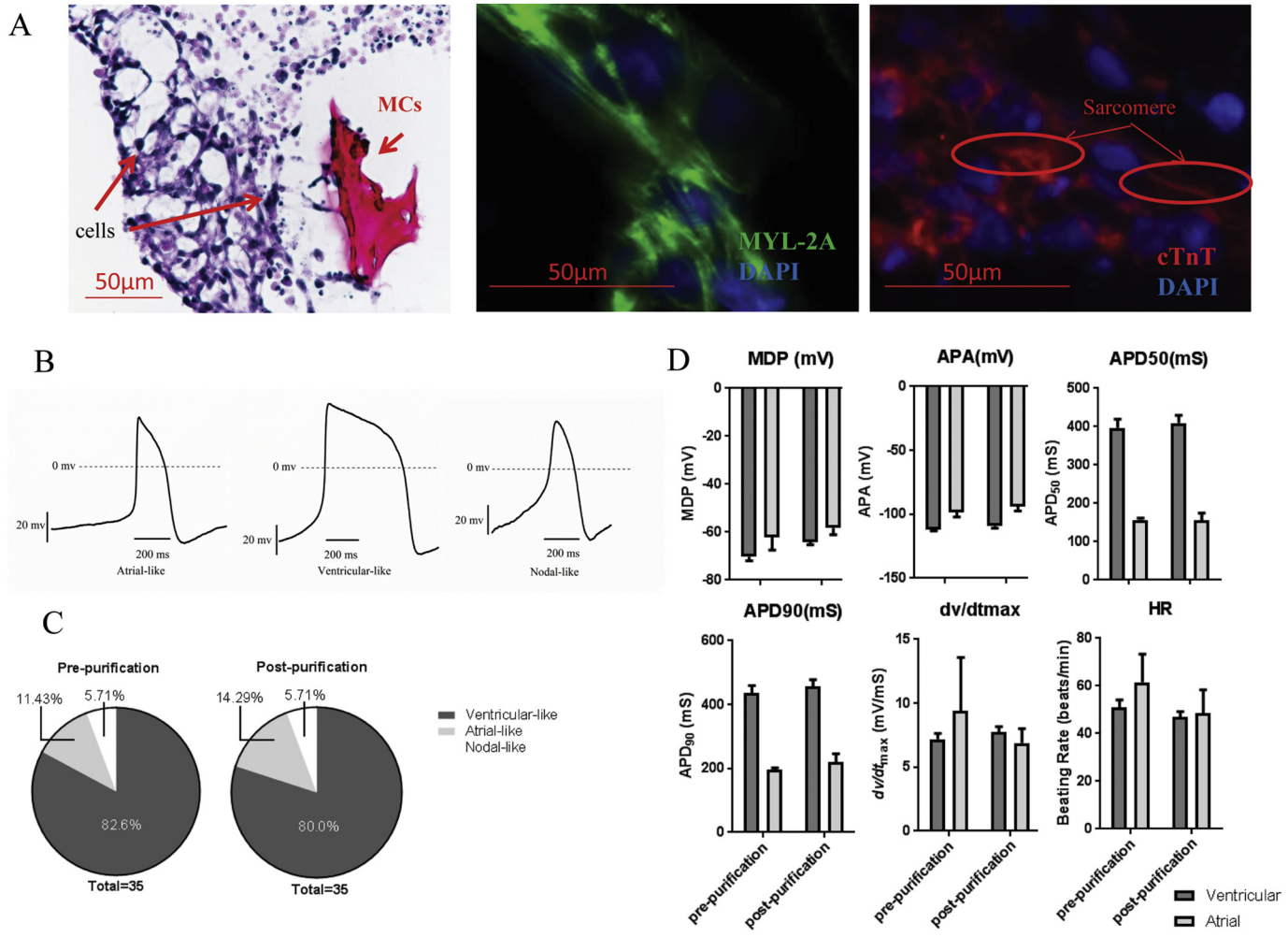


Fig. 6. Characterization of CMs obtained from the integrated expansion, differentiation and purification process.
HES-3 generated CM/MC aggregates were further analysed by microscopy using H&E staining to displays viable cells around the MCs and cTnT and MYL-2A to display cardiac proteins (A). Ventricular-like, Atrial-like and Nodal-like electrophysiological properties of the cells were measured (B). Electrophysiological properties of the CM pre- and post- purification was analysed by whole-cell patch clamp recordings (C). The maturation of the CM derived from cultures pre- and post-purification were measured by the Maximum Diastolic Potential (MDP), Action Potential Amplitude (APA), Action Potential duration at 50% and 90% of repolarization (APD50 and APD90), the Maximal rate of Depolarization (dV/dtmax), and the beating rate (HR) for both ventricular-like and atrial-like CMs (D).

6. Discussion

In this study, a three-stage protocol (expansion, differentiation, and purification) for efficiently differentiating human pluripotent stem cells to cardiomyocytes in a microcarrier-based stirred-tank reactor was developed. Using an intermittent agitation regime of 8/1 (which maintained DO at 30–100% air saturation without any oxygen starvation, Fig. 3A), significantly increases the growth of the differentiating cells (Fig. 3C) in comparison to cultures with 66/6 regime (which had a repeated hourly oxygen starvation phases during the first 3 days of differentiation; Fig. 3A). The availability of oxygen within a cell aggregate is affected by the aggregate size, impacting the cell expansion of the differentiating cells (Kinney et al., 2011). Thus the smaller and more uniform aggregates obtained in the 8/1 agitation regime can have additional beneficial effect on oxygen supply to the cells.

In order to meet the demand of CMs for cell therapy, several groups have tried to develop scalable culture systems for the large-scale production of human pluripotent stem cells (hPSC)-derived CMs using three-dimensional culture systems (Chen et al., 2015; Fonoudi et al., 2015; Fuerstenau-Sharp et al., 2015; Kempf et al., 2014; Lecina et al., 2010; Ting et al., 2014), see Table 3. Although suspended EB or aggregate cultures have the ability to be scaled up volumetrically, generating EB or aggregate structures is problematic though. It requires extensive cell handling as the cells need to be dissociated and re-aggregated to the appropriate size, in order to create efficient EBs for further differentiation. This may cause difficulties in maintaining reproducibility and cell viability (Placzek et al., 2009). The aggregates generated in the present integrated platform are uniform in size ($250 \pm 0.71\ \mu m^2$, Fig. 3D) and their generation does not require extensive cell handling that is required for EBs generation. Moreover, by comparing the yields of CM/hPSC seeded at the beginning of the hESC expansion, our present platform can produce high CM/hPSC yield (22 CM/hPSC), which is at least 4-fold more in comparison to the other groups reported (ranged from only 1–5 CM/hPSC, see Table 3). This difference in CM yields is mainly attributed by the high proliferative efficiency in the initial hPSCs expansion phase (~18-fold increase) in our platform, whereas there was only 1–5 fold expansion in other platforms. Herein, this is the first report demonstrating the generation of cardiomyocytes in scalable one unit stirred microcarrier culture which integrates expansion of pluripotent cells and differentiation, and purification of cardiomyocytes in one continuous process. Our microcarrier-based system is overall more cost-effective and less labour intensive than other platforms, which involved spontaneous EB formation.

Table 3
Summary of studies investigating the scalable cardiac differentiation protocols.

		Lecina et al., 2010	Tohyama et al., 2013	Ting et al., 2014	Kempf et al., 2014	Fuerstenau-Sharp et al., 2015	Chen et al., 2015	Fonoudi et al., 2015	Present work
	Platform	Microcarriers	EBs	Microcarriers	Aggregates	Aggregates	Aggregates	Aggregates	Microcarriers
	Format	Spinner flask (50 mL)	Petri dishes	T25 flask (33 mL)	Bioreactor (100 mL)	T75 flask (15 mL)	Spinner flask (1000 mL)	Spinner flask (100 mL)	Spinner flask (50 mL)
Cell Expansion	Cell density ($\times 10^6$ cells/mL)	1.5–2.2	ND	3.74 ± 0.55	~0.6	ND	~0.4	0.8–0.9	3.50 ± 0.51
	Expansion fold	2.3–3.4	ND	18.89 ± 2.82	1.8	ND	~0.5	4–4.5	17.51 ± 2.55
Cell differentiation	Cell density ($\times 10^6$ cells/mL)	~3	0.63 ± 0.23	2.45 ± 0.12	0.4–0.5	2.2–3.4	1.4 ± 0.4	0.8–0.9	2.31 ± 0.47
	Expansion fold	~3	ND	2.44 ± 0.15	1.2–1.5	2.2–3.4	ND	ND	2.31 ± 0.47
Purification	Method	ND	Lactate	ND	ND	MACS	ND	ND	Lactate
	Purity	15.73 ± 3.32% SA^+, MHC^+	~99% cTnT	65.73 ± 10.73% $cTnT^+$	85% SIRPα	68.3%–82.0% $cTnT^+$	> 90% cTnT	~90% $cTnT^+$ and MHC^+	72.5%–83.1% $cTnT^+$
	CM density ($\times 10^6$ CM/mL)	~0.2	0.47 ± 0.18	1.61 ± 0.36	0.34–0.43	0.45–0.72	1.3 ± 0.4	1–0.8	1.38 ± 0.22
	CM lost during purification	ND	~26%	ND	ND	~80%	ND	ND	~20%
CM/hPSC input		0.33	0.11	31.75 ± 9.74	0.72–0.9	2.2–3.4	~4.6	1–1.25	21.9 ± 3.3

Note: cTnT – cardiac troponin-T; SA – sacromeric; MHC – myosin heavy chain; SIRPα – signal-regulatory protein alpha.

Hydrodynamic effect in hPSC cultures, in general, have been reported to decrease pluripotency markers (such as Nanog; Fig. 1C) over time (Sargent et al., 2010) and enhance mesoderm differentiation through the modulation of Wnt/β-catenin pathway (Lei et al., 2014). Here, hydrodynamic conditions generated by different agitation regimes - continuous, intermittent 66/6 and 8/1 - influenced the temporal gene expression pattern of differentiation markers (Fig. 2C), and modulated the relative percentage of cells expressing cTnT and MF20 markers (Fig. 2B & Fig. 3C), suggesting that not all hydrodynamic environments affect CM differentiation in the same way. Hydrodynamics affected the relative magnitude of expression of differentiation genes (Fig. 2C). Two distinct Mesp-1 expression patterns in the differentiating cells were observed between continuous cultures and intermittent cultures (Fig. 2C). Upregulation of Mesp-1 was more rapid and higher in continuous cultures preceding expression in intermittent cultures during the first 3 days of differentiation. Mesp-1, a basic helix-loop-helix transcription factor, is one of the earliest markers for the vertebrate cardiovascular development (Saga et al., 2000). Reports showed that Mesp-1 acts as a master regulator during cardiovascular specification (Bondue et al., 2008). It induces features of epithelial-to-mesenchymal transition (EMT), as evident by the loss of the expression of the epithelial marker E-Cadherin (consistent with our data in Fig. 2C) in differentiating ESCs and promotes mesoderm towards a restricted set of cardiovascular fates including cardiomyocytes, smooth muscle cells, and endothelial cells (Lindsley et al., 2008). However, depending on the cellular environment, Mesp-1 also directs cells towards other mesodermal, namely haematopoietic and skeletal myogenic lineages (Chan et al., 2013). They showed that early expression of high levels of Mesp-1 induces haematopoietic differentiation, rather than cardiac differentiation. Therefore, we speculated that the high expression of Mesp-1 in continuous cultures during the first 24 h drives the differentiating cells primarily to the haematopoietic lineage, not promoting them to cardiomyocytes, which ultimately failed to form cardiomyocytes (low percentages of cTnT and MF20 were achieved at the end of the differentiation process). This speculation is further confirmed by the temporal expression of RUNX1 in continuous agitation culture, which is required for endothelial to haematopoietic cell transition (Chen et al., 2009).

To further increase the hPSC-derived CM production and purification using stirred suspension systems, we explored the impact of ascorbic acid in media, profile of agitation in stirred flasks and lactate purification method on CM differentiation. Media supplements with ascorbic acid have been shown to influence cardiac lineage commitment (Cao et al., 2012; Takahashi et al., 2003). Recent studies (Cao et al., 2012) have shown the importance of ascorbic acid in increasing the efficiency by affecting MEK/ERK signaling. Moreover, by combining the application of BMP4, CHIR99021 and ascorbic acid, they were successful in the production of cardiovascular progenitors from hPSCs (Cao et al., 2013). Similar to our results, the addition of ascorbic acid in the differentiation medium increased CM yields of 38.42% (Fig. 1), but did not affect the cell growth in the expansion phase in mTeSR1 medium.

Even if the protocols produce CMs with high efficiency, it is important to develop purification methods that enable the generation of pure CMs populations for clinical application. There are several approaches available for obtaining enriched cardiomyocyte populations from human hPSCs – antibody-based, physicochemical-based, biophysical-based and metabolic based (Ban et al., 2017; Schwach and Passier, 2016). Here, we integrated the metabolic based approach into our spinner platform to select CMs from heterogeneous cultures, by eliminating non-CMs cells, using lactate-rich and glucose-depleted medium. It simply takes advantage of the different metabolic signatures of cardiomyocytes compared to other cells (Hemmi et al., 2014; Tohyama et al., 2013). Simply by changing the differentiation medium to medium without glucose and containing ascorbic acid and lactate, CM's purity increased from ~50% to over 80% (Table 2B & C) after 5 days of cultures. By adding lactate and removing glucose in the purification media, this unique metabolic property of CM is a non-invasive approach to purify cells from PSC and other cell population (Hemmi et al., 2014; Tohyama et al., 2013). High purity CMs can be obtained by other

methods such as affinity-based or physicochemical-based, however these methods has the disadvantages of being expensive and leading to high loss of CMs during the purification process (Dubois et al., 2011; Fuerstenau-Sharp et al., 2015). Fuerstenau-Sharp et al. (2015) showed that although the CM purity increased from 36% to 89% by using antibody-based positive selection with either SIRPA (signal-regulatory protein alpha) or VCAM1 (vascular cell adhesion molecule 1), > 80% of $cTnT^+$ cells were lost during the purification procedure.

During lactate purification, the drop in the total cell number is hypothesized to be due to the death of non-CM cells in the culture, as they are unable to utilize lactate as a source of energy and anabolic precursor (Tohyama et al., 2013). This is confirmed in the kinetic experiment comparing purification media with different concentrations of lactate and glucose. The presence of glucose in the media leads to an increase in the total cell count but a decrease in the CM yield and purity, which confirms that non-CM cells are still able to grow in this media condition. A higher concentration of glucose present in the media produced the highest total cell count with the lowest CM yield, which means more glucose allowed more non-CM cell growth. In contrast, the media containing only lactate leads to a decrease in non-CM cells while maintaining the CM yield with high purity. Notably, the presence of even small amounts glucose (200 mg/L) in the lactate-rich purification medium prevents the CM purification process (Table 1). This observation in the reduction of non-CM cells in media containing only lactate is confirmed in the paper published by Tohyama et al. (2013).

To optimize the purification process, the timing for lactate introduction is also a crucial step. At the different time points where lactate purification is being initiated, it was observed that it can cause drastic changes in CM quantity and quality. When lactate purification was initiated after 7 days of differentiation, a much lower CM purity and CM cell count was achieved as compared to when purification was initiated on Day 10 and Day 12 (Fig. 5). Lundy et al. (2013) suggested that prolonged duration in culture may be required to approach the maturation *in vitro* that is usually achieved by the human heart which requires over ~40 weeks in the gestational period and further maturation for several years after birth. Breckwoldt et al. (2017) mentioned that the first spontaneous beating is generally observed by Day 8 to Day 10 of differentiation. Hence at least 10 days for cardiomyocytes differentiation is crucial to achieving a higher purity and quantity of CMs after purification.

In conclusion, a 3D suspended MC spinner platform that integrates hESC expansion, CM differentiation and purification has been developed and shown to be capable of producing high quantities of CMs ($201.7 \pm 75.7 \times 10^6$ to $242.3 \pm 49.6 \times 10^6$ cTnT positive CMs/production run that started with an initial of 50 mL of hESC culture). In the same way, a total of $1198.75 \pm 57.8 \times 10^6$ cTnT positive CMs/production run with a purity of 79.53 ± 6.65% was produced that started with an initial of 250 mL of HES-3 volume (data not shown). Further extensive investigation of scale up parameters on controlled bioreactors will be done in the future. This study describes a robust and scalable production and purification of CMs from hPSCs on microcarriers in a stirred suspension culture. Depending on the size of the bioreactor, large quantities of cardiac cells can be generated to meet clinical demands.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2018.07.020.

Disclosure statement

The authors declare no commercial or financial conflict of interest.

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