A Universal and Robust Integrated Platform for the Scalable Production of Human Cardiomyocytes from Pluripotent Stem Cells

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Running Title:

Scalable production of hPSC-derived cardiomyocytes

Footnote:

The Victor Chang Cardiac Research Institute does not engage in, nor does it condone, the destruction of human embryos for research. Its contribution to this study was limited to work on human induced pluripotent stem cells.

AUTHOR CONTRIBUTIONS

Hananeh Fonoudi and Hassan Ansari: Designed experiments and drafted sections of manuscript, performed cell culture, real-time PCR analysis, imunocytofluorescence and flow cytometry.

Saeed Abbasalizadeh: Designed all experiments and drafted sections of manuscript.

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Abstract

Recent advances in the generation of cardiomyocytes (CMs) from human pluripotent stem cells (hPSCs) in conjunction with promising outcomes from pre-clinical and clinical studies, have raised new hopes for cardiac cell therapy. Here, we report the development of a scalable, robust, and integrated differentiation platform for large-scale production of hPSC-CM aggregates in stirred suspension bioreactor as a single-unit operation. Precise modulation of the differentiation process by small molecule activation of WNT signaling followed by inactivation of TGF-β and WNT signaling, and activation of SHH signaling in hPSCs as size-controlled aggregates led to generation of approximately 100% beating CMs spheroids containing virtually pure (~90%) CMs in 10 days. Moreover, the developed differentiation strategy was universal as demonstrated by testing multiple hPSC lines (5 hESC and 4 hiPSC) without cell sorting or selection. Produced hPSC-CMs successfully expressed canonical lineage-specific markers and showed high functionality as demonstrated by microelectrode array and electrophysiology tests. This robust and universal platform may provide a valuable tool for mass production of functional hPSC-CMs as perquisite for realizing their promising potential for therapeutic and industrial applications including drug discovery and toxicity assays.

Keywords: Human pluripotent stem cells; ES; iPS; hPSC; Cardiomyocytes; Directed differentiation; Cell therapy; Small molecules; Bioreactor.

Introduction

Human pluripotent stem cells (hPSCs) including human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) can be considered as an unlimited source for production of cardiomyocytes due to their self-renewal and directed differentiation capabilities[1, 2]. Several approches including embryoid body formation [3], co-culture [4], cytokine-directed differentiation [5] and protein transduction [6] have been introduced to generate hPSC-derived cardiomyoctes since the first report in 2001. In spite of significant methodological advances, the hPSC-based production platforms still suffers from critical disadvantages including high cost, low efficiency, and reproducibility. This limits the application of hPSCs derived cardiomyocytes for clinical and industrial applications such as drug discovery and toxicity testing[7].

Indeed, most of the promising applications of hPSCs-CMs including those that are more close to commercialization such as high-throughput drug screening/toxicity testing followed by clinical applications, necessitate the production of massive numbers of pure and functional CMs [8]. Therefore, developing robust and affordable technologies for large-scale expansion of hPSCs and their integrated differentiation into cardiomyocytes in scalable culture systems would largely facilitate their commercial applications.

To date, a number of different protocols and technologies have been introduced for the production of CMs from hPSCs with the aim of optimizing different aspects of their bioprocessing including culture conditions that favor the production of mature and fully functional CM, and robust, integrated and scalable differentiation and purification methodologies [9, 10].

To develop fully defined culture conditions for generation of hPSC-CMs, exploring novel and efficient small molecules (SMs) for chemically induced cardiac differentiation has recently

emerged as a viable alternative to recombinant cytokines and unknown factors in serum [11-13]. Indeed, manipulation of signaling pathways required for normal heart development has guided the development of efficient hPSC-CMs differentiation protocols [9, 14-16].

On the other hand, current experimental methods for the differentiation of hPSCs often rely on the production of heterogeneous cellular aggregates termed "embryoid bodies" (EBs), or 2D small-scale static cultures [17-19]. These protocols are typically not scalable and/or result in generation of EBs with highly heterogeneous size and low CM yield. In order to establish more robust bioprocesses, different approaches such as microwell-mediated control, microprinting technologies [20-23] and microcarrier cultures [24] have been applied to address these issues. However, these techniques suffer from limited differentiation potential, scalability (microwell-mediated control, hanging drop, and microprinting technologies), universality and/or reproducibility due to the differentiation protocol itself or the low throughput of the used methods like forced aggregation techniques before transferring cells to dynamic culture conditions. In addition, most of the protocols depends on using expensive and complex media/reagents (mTeSR1 or StemPro-34) or microcarriers for expansion of hPSCs and their directed differentiation to cardiomyocytes [25, 26].

Recently, efforts have been made to develop scalable culture systems for large-scale production of hPSCs-CMs under 3D culture conditions. Kempf et al. tried to generate hPSC-CMs in a 100 ml stirred suspension bioreactor with batch and perfused modes using mTeSR medium for the hPSCs expansion phase and RPMI/B27 medium for the differentiation phase. However, batch cultures failed to generate contracting EBs [27] and perfused cultures resulted in contracting EBs with heterogeneous size (350–600 μm diameter) which may limit their future application for clinical and industrial use.

In a previous study, we developed a robust and cost effective culture system for mass production of size-controlled hPSCs aggregate cultures in stirred suspension bioreactors. Our protocols have paved the way for mass production of these unique cells under xeno-free conditions with superior scalability (for review see [28]). Subsequently, we have shown that this platform can be easily integrated for large-scale generation of hepatocyte-like cells that improved hepatic failure in an animal model after transplantation [29, 30].

In this report, we have used the a platform for development of an integrated, simplified process for large-scale production of highly homogenous hPSC-CM aggregates in a cost effective single-unit operation with high efficacy, reproducibility and universality. This scalable production system can be easily integrated with an efficient scalable purification system including culture-based methods such as those using lactate-enriched medium for selective purification of CMs, to generate a highly pure population of cardiac cells for biomedical applications [31]. Development of such integrated platforms can be considered as an important step toward commercialization of hPSCs-CM-based technologies for clinical, pharmaceutical, tissue engineering, and *in vitro* organ development applications.

Materials and Methods

Generation of fibroblast cultures

Ethics approval was obtained from the Sydney Children's Hospital Network Human Research Ethics Committee, reference number HREC/10/CHW/44. Suitable participants were selected from the Kids Heart Research DNA Bank and consent was obtained. Skin biopsies were performed by cardiothoracic surgeons and sampled from the upper arm of the participants. Fibroblasts were cultured from skin biopsies to establish cell lines for each participant.

Culture of hPSCs as aggregates

hESC lines (RH5, RH6, R725.1, R661.5, and R662.2) [32, 33] and hiPSC lines (VC645-9, VC913-5, VC618-3 and VC646-1), the latter generated at the Victor Chang Cardiac Research Institute, Australia, were used in this study. hPSCs were expanded by a previously described suspension culture method [29, 34]. Briefly, to initiate suspension cultures, 2×10^5 viable cells/ml were transferred to non-adhesive bacterial plates (60 mm; Griner, 628102) in 5 ml of hESC conditioned medium containing 100 ng/ml bFGF (Royan institute). The cells were incubated under standard conditions (37°C, 5% CO2 and saturated humidity). The medium was renewed daily. hESC medium included Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Gibco, 21331-020) supplemented with 20% Knockout Serum Replacement (KOSR, Gibco, 10828-028), 2 mM L-glutamine (Gibco, 25030-024), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, M7522), 1% nonessential amino acids (Gibco, 11140-035), 1% penicillin and streptomycin (Gibco, 15070-063), 1% insulin–transferrin–selenite (Gibco, 41400-045).
Conditioned medium was prepared by overnight incubation of hESC medium (without bFGF) with confluent human foreskin fibroblasts in 75-cm^2 T flasks, previously inactivated by treatment with mitomycin C (Sigma-Aldrich, M0503).

Optimizing differentiation process for cardiac differentiation in static suspension culture

The hPSCs aggregates cultured in static suspension mode, i.e., non-adhesive bacterial plates, were directly differentiated into CMs for optimization trials. In order to induce CMs differentiation from hPSCs in suspension culture systems, 3-, 5- and 7-day-old hPSCs size-controlled spheroids (average size: 90±30, 175±25 and 250±32 μm, respectively) were treated

for 24 h in differentiation medium (RPMI 1640, Gibco, 31870-022) supplemented with 2% B27 without retinoic acid (Gibco, 12587-010) or without insulin (Gibco, A18956-01), 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acids, 1% penicillin and streptomycin and different concentrations (3, 6, 9, 12 and 15 µM) of the SM CHIR99021 (CHIR, Stemgent, 041-0004) as glycogen synthase kinase (GSK3) inhibitor and canonical WNT/β-catenin pathway activator. The spheroids were washed with Dulbecco's phosphate-buffered saline (DPBS) and then maintained in fresh differentiation medium without SMs for one day. After one day, the medium was exchanged with new differentiation medium that contained 5 µM IWP2 (Tocris, 3533) as a WNT antagonist, 5 µM SB431542 (Sigma-Aldrich, S4317) as inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors, and 5 µM purmorphamine (Pur, Stemgent, 04-0009) as a SHH agonist. Spheroids were cultured for two days in this medium. After washing the spheroids with DPBS, fresh differentiation medium without SMs was added and refreshed every 2-3 days until the end of the differentiation process at day 30.

Integrated differentiation of hPSCs toward CMs in a stirred suspension bioreactor

In order to develop an integrated differentiation process to generate human CMs from hPSCs in a scalable manner, we expanded hPSCs as a single cell inoculated suspension culture in 125 ml spinner flasks (Cellspin; Integra Biosciences, Switzerland) with 100 ml working volume at 40 rpm agitation rate as previously described [29]. Briefly, to initiate the dynamic cultures, 2×10^5 cells/ml were transferred to 100 ml hPSC medium, conditioned on human foreskin fibroblast and contained freshly added 100 ng/ml bFGF [29], in the stirred bioreactor. Cell aggregates were treated with 10 µm Rho activated kinase inhibitor (ROCKi, Y-27632, Sigma-Aldrich, Y0503) 1h

prior to enzymatic dissociation with Accumax™ cell aggregate dissociation medium [Innovative Cell Technologies, Inc.; AM105]. The bioreactor was agitated at 35 rpm (increased to 40 rpm after 24 h) and medium refreshing began after 48 h of culture with the same medium without ROCKi. The seeded spinner flasks were incubated under standard conditions. After passages in dynamic suspension culture, the cells were cryopreserved as previously described [30] or induced for differentiation.

To induce CM differentiation from hPSCs in dynamic suspension culture, time points, differentiation media, and SMs were exactly similar to the static system with the exception of the addition of 0.1% poly vinyl alcohol (PVA, Sigma-Aldrich, 363073) for the first 48 h and 10 µm ROCKi for the first 24 h. In addition, aggregates were washed twice with 25 ml DPBS after mesodermal and cardiac induction steps by stopping agitation for 5-10 min and removing spent media containing SMs cocktails. After washing cardiac induced aggregates, fresh differentiation medium (100 ml) added to culture vessel without SMs and totally refreshed every 2-3 days until the end of the differentiation process at day 30.

Exploring the optimum hPSCs aggregate size for integrated cardiac differentiation

In order to achieve size-controlled hPSCs aggregates and subsequently hPSC-CMs with high differentiation efficacy, the RH5 cell line was cultured in dynamic suspension culture for 3, 5, and 7 days under optimal hydrodynamic culture conditions. The diameters of size-controlled aggregates generated in each culture were quantified using a phase-contrast inverted microscope (Olympus, CKX41) and Olysia Bioreport software. Then, hPSC aggregates of a defined size were transferred to differentiation media under static conditions to explore the optimum size or dynamic culture systems for differentiation.

Quantification of beating spheroids

We determined the cardiogenic differentiation efficiency by using an inverted cell culture microscope (Olympus, CKX41) to count the number of beating hESC and hiPSC spheroids throughout the experiment. The numbers of beating spheroids were normalized to the total numbers of spheroids at each time point. All quantification experiments and analyses were performed using at least three independent biological replicates.

RNA isolation and quantitative real-time polymerase chain reaction

We collected the hPSC spheroids at various time points in the differentiation process in both static and dynamic systems. Total RNA was extracted using the TRIzol reagent (Invitrogen). RNA quality and concentration were analyzed with a spectrophotometer (Biochrom, WPA). Possible genomic DNA contamination was removed by DNase I (Invitrogen) treatment for 15 min at room temperature after which 2 μg of total RNA was used for reverse transcription with an oligo $(dT)_{20}$ primer and Super Script III First-Strand Synthesis System (Invitrogen), according to the manufacturer's instructions. Quantitative RT-PCR was performed with the Power SYBR Green PCR Master Mix (Applied Biosystems) in triplicate for each sample and each gene. PCR conditions included denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min for 35 cycles, with a 72°C extension for 7 min at the end. Expression of genes of interest was normalized according to *GAPDH* expression. Relative gene expression levels were quantified by the 2(-Delta Delta C (T)) method. Primer sequences are listed in Supplementary Table S1.

In order to analyze quantitative RT-PCR data, we used R statistical language [35]. Principal component analysis (PCA) was performed on the scaled data. For the time-course analysis, genes were clustered according to expression values in different samples using a K-means algorithm. Visualization of data was performed using the R packages ggplot2 [36] and heatmap.

Flow cytometry

RH5 hESC spheroids were collected at different time points after differentiation initiation in static and dynamic systems, washed twice with PBS, incubated with 0.05% trypsin-EDTA (Gibco, 25300-054) at 37°C for 4-5 min, and then pipetted 5–12 times. After neutralizing trypsin activity by the addition of medium, the cell suspension was passed through a 40 µm filter mesh (BD Falcon, 352340) to remove clumps and undissociated spheroids. Following trypsinization and achievement of single-cell suspensions, cells were washed twice in ice-cold staining buffer (PBS supplemented with 1% heat-inactivated FBS, 0.1% sodium azide, and 2 mM EDTA) and fixed in high-grade 4% paraformaldehyde (PFA) for 15 min at 4°C. Cells were washed again with staining buffer, permeabilized with 0.2% (v/v) Triton X-100 in PBS for 20 min, and blocked for 15 min at 4°C with a combination of 10% heat-inactivated goat serum in staining buffer. Cells were incubated overnight at 4°C (or 30 min at 37°C) with the suitable primary antibodies (1:100) or appropriate isotype matched controls, then washed three times with staining buffer after which secondary antibodies (1:500) were added to the cells. After 45 min incubation at 4°C, cells were washed three times with staining buffer and analyzed by a flow cytometer (FACSCalibur, BD Biosciences) and flowing software version 2.5.1 (BD Biosciences). For each analysis, 0.5-1 $\times$ 10^6 cells were used per sample. All experiments were

replicated at least three times. Primary and secondary antibodies used for flow cytometry are listed in Supplementary Table S2.

Immunostaining and imaging

hPSC differentiated beating spheroids were collected at different time points after differentiation initiation in static and dynamic systems. The collected spheroids were washed twice with PBS and dissociated into single cells with 0.05% trypsin-EDTA (Gibco, 25300-054) at 37°C for 4-5 min. Individualized cells were cultured on gelatin-coated chamber slides (Nunc, 177437) in RPMI/B27 medium. After 5 days the attached cells were washed once with PBS, fixed with 4% (w/v) PFA at room temperature for 15 min, washed once with washing buffer (PBS/0.1% Tween 20), permeabilized with 0.2% Triton X-100 in PBS for 15 min, and blocked with 5% (v/v) goat serum for 1 h. Primary antibodies diluted in blocking buffer (1:100) were added to the cells followed by an overnight incubation at 4°C. Following incubation the cells were washed three times with washing buffer, each for 5 min. Secondary antibodies diluted in blocking buffer (1:500) were added to cells, after which they were incubated for 1 h at room temperature. Cells were subsequently washed three times with washing buffer, then covered with a Vectashield mounting medium that contained DAPI (Vector Laboratories, Inc.). Imaging was performed on an upright confocal microscope (Zeiss LSM700).

For immunohistochemistry analysis, the beating RH5 hESC spheroids were collected, washed with PBS, fixed with 4% (w/v) PFA at room temperature for 15 min, and prepared for paraffin-embedded tissue blocks. Paraffin-embedded spheroids were cut into 6 µm sections using a microtome (MICROM HM325) and kept at room temperature until use. For staining, we de-waxed and hydrated the spheroid section slides followed by heat-mediated antigen retrieval using

a Dako target retrieval solution (Dako, S2367). Permeabilization, blocking steps, and incubation with primary and secondary antibodies were performed as described above for individualized cultured cells. Primary and secondary antibodies used for cultured cells and staining the spheroids are listed in Supplementary Table S2.

Microelectrode array recording

We characterized the functional properties of hESC spheroid-derived CMs by performing an extracellular recording of field potentials (FPs) using a microelectrode array (MEA) data acquisition system (Multi Channel Systems, Reutlingen, Germany). The MEA plates contained a matrix of 60 titanium nitride electrodes (30 μm) with an inter-electrode distance of 200 μm. MEA plates were sterilized and hydrophilized with FBS for 30 min, washed with sterile water and coated with 0.1% gelatin for 1 h. For this analysis, areas of plated beating spheroids were mechanically dissected and plated on the middle of a sterilized MEA plate in medium that contained 20% FBS. On the day of the experiment, coated MEAs were connected to a head stage amplifier. Extracellular potentials were sampled at 50 KHz and all recordings were performed at 37°C. Recordings were performed for 100 s at baseline and at 5 min after drug application. Field potential (FP) signals were analyzed for FP duration (FPD, defined as the interval between FPMin and FPMax), interspike intervals (ISI) and beating frequency. Data were analyzed using AxoScope software (Molecular Devices).

Patch clamp electrophysiology

Action potentials were recorded from spontaneously beating hESC- or hiPSC-derived CMs using current clamp in the whole cell patch clamp configuration. Beating spheroids were dissociated

into single cells using 0.05% trypsin-EDTA (Gibco, 25300-054) at 37°C for 4-5 min. Single beating cardiomyocytes were plated onto glass coverslips coated with ECM Gel (Sigma-Aldrich, E1270) and maintained at 37°C. Prior to experimentation, the coverslips were transferred to a recording chamber mounted on the stage of an Olympus inverted microscope (Olympus, CKX41). The extracellular bath solution within the chamber contained: 150 mM NaCl, 5.4 mM KCl, 15 mM HEPES, 15 mM D-glucose, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$; adjusted to pH7.4 with NaOH. Recording pipettes were pulled from borosilicate glass capillaries using a Sutter Instruments P-97 horizontal puller and had tip resistances between 3 and 6 MΩ. The pipette solution contained: 150 mM KCl, 10 mM NaCl, 2 mM $CaCl_2$, 5 mM EGTA, 10 mM HEPES, and 5 mM MgATP; adjusted to pH7.2 with KOH. Data were acquired using a multiclamp 700B amplifier (Axon Instruments), a Digidata 1440 analog-to-digital (A/D) board and pClamp10 software (Axon Instruments), at a sampling frequency of 10 kHz and low-pass filtered at 2 kHz. Data analysis was performed using Clampfit 10 (Axon Instruments) and Prism 6 (Graphpad) software.

Statistical analysis

All quantifications have been performed using at least three independent replicates. Data are presented as means ± SD in which data with symmetrical (normal) or non-symmetrical distributions were analyzed with one-way ANOVA followed by the post hoc LSD or Dunnett's significant difference tests. $P<0.05$ was considered statistically significant (a: $0.01<p\text{-value}<0.05$; b: $0.001<p\text{-value}<0.01$; c: $p\text{-value}<0.001$).

Results

Modulation of WNT, TGF-β, and SHH signaling pathways to enhance cardiac differentiation

In order to develop an efficient protocol for large-scale generation of CMs from hPSCs, we attempted to manipulate the most important cardiogenic signaling pathways by using SMs in adherent culture. First, we optimize chemically-induced CM differentiation methods using recently reported SM cocktails [14, 15]. After several optimization trials employing multiple different combinations of SMs and treatment durations, we determined that the most effective protocol for CM differentiation was exposure of hESC to 12 μM CHIR for one day, followed by a one day rest period in the same medium without SMs. Subsequently, IWP2, SB431542 and Pur (5 μM each) were used for an additional two days. The SMs inhibited WNT/β-catenin and TGF-β, and activated the SHH signaling pathways, respectively. This method led to the appearance of beating clusters in the adherent cultures at days 7-10 after the onset of differentiation (data not shown).

In order to transform the optimized adherent culture protocol to scalable suspension culture conditions, we induced 7-day spheroids of hPSCs that had been generated in low-attachment dishes under static suspension culture conditions with the optimized protocol (Figure 1). For differentiation, cells were treated as for the adherent culture protocol.

Daily analysis of differentiation in spheroids showed that the first beating appeared after 10 days of differentiation induction (Figure 1B). To determine the optimal size of spheroids for CM differentiation, 3-, 5- and 7-day hESC spheroids (average size: 90±30, 175±25 and 250±32 μm, respectively) generated in dynamic suspension culture were used for differentiation induction in the static suspension system. In the case of 3-day spheroids, cell viability was significantly decreased; spheroids became disrupted and finally dispersed after CHIR treatment (data not

shown). We excluded this group from further experiments. We observed that approximately 50% of the 5-day spheroids started beating only 7 days after differentiation induction; approximately 100% of spheroids formed beating structures after 10 days of differentiation induction and maintained spontaneous contractile activity for at least 30 days in culture (Figure 1B). The average size of the 5-day spheroids was 175±25 μm; therefore, this was chosen as the optimal size for the subsequent experiments of this study.

Optimization of chemically induced cardiomyocyte differentiation in static suspension system

Different concentrations of CHIR have been reported which favor mesendoderm induction. The 3D structure of spheroids might affect the diffusion rates of CHIR throughout the cell aggregates, which could in turn result in inefficient and heterogeneous differentiation. Indeed, the sensitivity of cells in these compact structures might be completely different to that of cells in monolayer adherent culture. Therefore, to explore the optimal concentration of CHIR for induction of hPSC aggregates, we treated the 5-day aggregates with different concentrations of CHIR (3, 6, 9, 12 and 15 μM) and spheroids were assessed after 10 days of differentiation.

Our data showed that by increasing CHIR concentration, the percentages of beating spheroids also increased. Approximately 100% of the spheroids began beating at both 12 μM and 15 μM CHIR (Figure 1C). Flow cytometry analysis showed that αMHC expression increased and reached a plateau (>90%) after treatment with 12 μM and 15 μM CHIR (Figure 1C); this was also supported by the quantification of beating. These results suggested CHIR at 12 μM as the optimal concentration for mesoderm induction. This concentration was used in the first step of our differentiation protocol in suspension cultures.

We sought to determine if manipulating one or two signaling pathways was sufficient to induce efficient CM differentiation or whether the complete cocktail of three chemicals (IWP2, SB431542 and Pur) was necessary. We examined different combinations of these three chemicals and calculated the number of beating spheroids after 10 days of differentiation (Figure 1D). The results indicated that treatment of cell aggregates with SB431542 or Pur resulted in the formation of only few beating spheroids, whereas IWP2 treatment led to higher numbers of beating spheroids (up to 20%), which confirmed a role for WNT signaling in cardiogenesis in this static suspension system. The combination of SB431542 and Pur resulted in a lower percentage of beating spheroids compared to IWP2 and the other two-chemical combinations. The combination of three chemicals dramatically increased the number of beating spheroids to approximately 100%; hence, this was identified as the most effective combination at this step for CM differentiation in the suspension culture condition. Flow cytometry analysis of αMHC^{+} cardiac cells at day 10 after differentiation initiation supported the beating results and revealed that a large population of CMs (>90% positive for αMHC) formed when using all three chemicals (Figure 1D). These results suggested that inhibition of WNT signaling was necessary at this step for CM differentiation in suspension cell aggregates. To achieve an efficient differentiation process, inhibition of TGF-β signaling and activation of SHH signaling was also required.

Gene expression pattern of cardiogenic genes in a static suspension system

We determined the expression profiles of cardiac lineage-specific genes throughout differentiation by performing quantitative RT-PCR (qPCR) on spheroids collected at different time points after differentiation initiation (days 0, 1, 2, 4, 6, 8, 10, 20, and 30). Rapid induction

of *T (Brachyury),* a mesodermal marker, and *Mesp1,* a marker for the earliest steps of cardiovascular progenitor cell specification, was observed at the first two days of differentiation, confirming the role of WNT signaling in mesoderm lineage specification (Supplementary Figure 1). Downregulation of these genes occurred simultaneously with significant upregulation of later cardiac progenitor transcription factors (*HAND1, TBX5, ISL1, MEF2C, NKX2-5*) at day 4 of differentiation. The expression of *GATA4* increased earlier compared to other cardiogenic transcription factors and displayed a steady level of expression over time. Expressions of *cTNT*, *α-MHC* and *β-MHC* (structural protein coding genes) were upregulated at day 6 of differentiation and maintained until the end of the experiment.

Flow cytometry analysis of differentiated cells at day 15 showed that the majority of individualized cells (up to 90%) were $cTNT^+$, while endothelial cells (vWF^+) and vascular smooth muscle cells (αSMA^+) represented a small proportion of the differentiated cells (~3% and ~8%, respectively; Supplementary Figure 2).

Taken together, these results show that CM differentiation in the static suspension system passed through the main steps of cardiogenesis seen in normal heart development as well as in embryoid body- and monolayer-based differentiation methods.

Validation of optimized cardiomyocyte differentiation for different hPSC lines in the static suspension culture system

To test if our developed protocol supported robust CM differentiation in different hPSC lines, we differentiated five hESC and four hiPSC lines in the static suspension system using an optimal induction procedure. Analysis of the percentage of beating spheroids showed that almost all lines started spontaneous beating at day 7 of differentiation, which increased rapidly to a plateau with

nearly 100% beating spheroids at days 10-15 (Figure 2A). These data demonstrated the reproducibility and universality of our protocol for all tested hPSC lines. Flow cytometry analysis of dissociated beating spheroids demonstrated that approximately 90% of cells were $cTNT^+$ in four evaluated cell lines at day 15 of differentiation (Figure 2B). Subsequent experiments were performed with the RH5 hESC line.

Integrated generation of human CMs in a stirred suspension bioreactor

In order to develop an integrated platform for large-scale production of human CMs, we employed our optimized static suspension differentiation strategy in a stirred bioreactor with a 100 ml working volume. Five-day hPSC spheroids that were 175±25 μm in diameter were induced in a spinner flask by replacing hPSC expansion medium with the same volume of differentiation medium (100 ml) for differentiation induction (Figure 3A). After several rounds of experiments, we found that addition of 0.1% PVA, and 10 μM ROCK inhibitor Y-27632 were essential for the first two days of differentiation culture to increase cell viability and spheroid integrity (data not shown). The efficiency of CM differentiation was determined by calculating the number of beating spheroids in the first two weeks of differentiation (Figure 3B). Interestingly, when compared with the static differentiation system, the first spontaneous beating appeared at day 7. The percentage of beating spheroids increased progressively and reached a plateau with approximately 100% beating spheroids by day 10 (Supplementary Video 1). Immunostaining for cTNT, MLC2a, and NKX2-5 in sections of beating spheroids collected at day 10 showed cytoplasmic- and nuclear-localized immunolabeling for cTNT and MLC2a, and NKX2-5, respectively, which indicated the efficiency of CM differentiation (Figure 3C). Furthermore, immunostaining for MLC2a and co-staining for NKX2-5 and cTNT in cells from

both hESC and hiPSC lines demonstrated the nuclear accumulation of NKX2-5 and defined sarcomeric structures in differentiated CMS (Figure 3D).

Cardiac specific gene expression pattern and cardiac lineage induction in a dynamic suspension system

We examined pluripotency and cardiac-specific gene expression patterns and transcript enrichment in hPSCs during CM differentiation in a dynamic culture system by performing qRT-PCR at eight time points (Supplementary Figure 3). The results showed that the pluripotency genes *OCT4* and *NANOG* were significantly downregulated and mesodermal marker *T* was upregulated after differentiation induction. The highest level of *MESP1* expression was observed at day 2. Expressions of cardiac progenitor markers *c-KIT, ISL1,* and *PDGFR-α* increased during differentiation with the highest level of expression at day 8. Late cardiac progenitor markers *NKX2-5, TBX5, MEF2C,* and *GATA4* were upregulated exactly after differentiation induction, peaked at days 6-8, and remained at a steady high level of expression until the end of the study (day 30). Cardiac structural and maturation markers such as *cTNT*, *αMHC,* and *MLC2v* began expression at day 6 and reached a plateau with sustained expression until the end of study at day 30.

We grouped pluripotency and differentiation genes into three clusters according to their expression pattern (Figure 4A). Cluster one includes pluripotency genes (*OCT4* and *NANOG*) and mesodermal marker *T*, with all showing downregulation during CM differentiation. The genes in cluster two showed a later peak after differentiation induction, followed by downregulation over the differentiation process. Cluster three included genes that encode CM specific transcription factors and structural proteins. These genes showed an upregulation and

then sustained expression throughout CM differentiation. Replicated samples were clustered together in principal component analysis (PCA) that showed a clear roadmap of differentiation (represented by an arrow in Figure 4B, left). This map starts from day 0 (hPSCs) and terminates at day 30. While pluripotency genes *NANOG* and *OCT4* are located in the same direction of day 0 replicates, *MLC2v* is found at the end of differentiation roadmap. These analyses demonstrated the quality of the obtained results and reproducibility of the experiment.

To quantify the percentage of cardiac lineage cells we performed flow cytometry analysis at different time points in which cardiac lineage genes showed highest level of expression (Figure 4C). T^+ mesodermal cells comprised a high fraction of spheroids cells (~90%) at day 2 of differentiation and this percentage decreased during differentiation. $MEF2C^+$ cells represented ~50% of total cells at day 6 which increased to approximately 80% at day 8 of differentiation. A rapid increase in the percentage of αMHC^+ was observed after day 6 which increased further to 85% by day 10. Taken together, these flow cytometry analyses corroborated the gene expression results discussed above and showed the stepwise induction of different cardiac lineage states in a dynamic differentiation system.

Electrophysiological properties of human CMs differentiated in a dynamic suspension system

We investigated the electrophysiological properties of hPSC-derived CMs using MEA and patch-clamp techniques. We sought to determine whether integrated differentiation in a dynamic suspension culture system will produce functional CMs. hPSCs spheroid-derived CMs in dynamic suspension system developed spontaneous electrical activity, as indicated by the typical extracellular field potentials (FPs) formed at different areas of beating spheroids. The presence of

a sharp and a slow component of the FP was noted (Figure 5A). The FPs could be divided into a rapid component that reflected depolarization, a plateau phase, and a slow component represented repolarizations [37]. We observed the chronotropic response of the spheroid-derived CMs following administration of the β-agonist isoproterenol (Iso). Application of 100 nM Iso resulted in a typical and comparable increase of the FP frequency compared to basal condition (Figure 5B). Moreover, the FP duration was significantly shortened in Iso-treated beating spheroids (525±5 at baseline vs. 485±6 at Iso treatment). Thus, the dynamic suspension culture system supported the development of spontaneous contractile activity and electrophysiological functionality.

We also studied action potentials in single beating cells using the whole cell mode of the patch clamp technique. A primary study of single beating cells at day 30 of differentiation showed nodal-like APs in the majority of patched cells (Supplementary Figure 4). This classification was based on the morphology of APs (Supplementary Figure 4 A), AP parameters (Vm<10V/s and APD90≤150) [38, 39] and mean beating rate >100 (Supplementary Figure 4B and 4C). The application of quinidine at 100 μM resulted in deceleration (Supplementary Figure 4D (b)) of spontaneous APs followed by complete abolition of beating, demonstrating the contribution of I_{Na} in depolarization phase of these APs. The cells showed partial recovery after washout (Supplementary Figure 4D (d)). Quinidine also caused prolongation of the AP duration, showing the contribution of hERG channels in the repolarization phase of these cells. The effect of quinidine was reversible, although it had not fully reversed after 5 minutes consistent with the slow dissociation kinetics for quinidine.

AP recording from single beating hPSC-derived CMs was also repeated at days 60 and 90 of differentiation. We could observe APs of the three main cardiac cell types in cells differentiated

from hPSCs in dynamic suspension system (Figure 6A-6C). Vm>10 (20.5±1.9 V/s) and APD90/APD50≤1.6 (1.6±0.09) was measured in morphologically ventricle-like APs which further confirmed their subtype (Figure 6A). Nodal-like APs showed Vm<10 (8.7±0.96V/s) and 1.6<APD90/APD50<2 (1.8±0.03) (Figure 6C). Atrial-like APs showed similar properties to ventricle-like APs, but with shorter AP durations (Figure 6B). Thus, integrated differentiation of hiPSCs in a dynamic suspension culture system supported the generation of different cardiac cell types.

Discussion

Effective cardiac cell therapies as well as development of pharmacology and toxicology screening platforms require large numbers of pure and functional cardiac cells that cannot be easily produced by the majority of current protocols for the generation of CMs from hPSCs. For example, to achieve an effective cardiac cell therapies 1-2 × 10^9 of hPSC-CMs are required for myocardial infarction (for review see [40]). Therefore, developing flexible and robust protocols and platforms for cost-effective, reproducible and large-scale generation of desired hPSC-derivative cells is necessary to realize the great potential of hPSC-CMs for clinical and commercial applications.

To date, different CM differentiation protocols have been reported that manipulated the WNT, TGF-β, and SHH signaling pathways by different protein factors, matrix components or SMs [14, 15]. However, most of the protocols suffer from large variations in CM differentiation efficacy between different cell types and lines, as experienced in our preliminary experiments. After numerous trials, we have developed a new procedure using CHIR (12 μM, WNT/β-catenin signaling activator) for one day to induce mesendodermal differentiation, followed by a one-day

rest period, and then 2 days of treatment with a SM cocktail comprised of IWP1 (WNT/β-catenin inhibitor), SB431542 (TGFβ receptor inhibitor) and Pur (SHH agonist) to generate CMs. It is known that WNT signaling has a biphasic role on cardiogenesis [41]. Addition of WNT protein or IWP2 can reduce endogenous WNT heterogenity and provide a condition for stable expansion and efficient differentiation of WNT ^{high} or WNT ^{low} hESC populations, respectively[42]. However, our differentiation method has eliminated the variability in CM differentiation between hPSCs lines, which have been reported in other related studies. This universality of production process will largely facilitate the future widespread application of the protocol and development of hPSC-CMs based therapeutic products.

More recently, another universal CM differentiation protocol for hiPSCs was developed using a chemically defined medium that consisted of three components - basal medium RPMI 1640, L-ascorbic acid 2-phosphate and rice-derived recombinant human albumin. This protocol resulted in generation of CMs with high efficacy and productivity. However, the proposed culturing platform was based on a 2D adherent culture on peptide modified surfaces which are very expensive and offering poor scalability [9].

To overcome the complexity and limited scalability issues of previously developed CM generation protocols, we have successfully developed a cost-effective and robust process that allows large scale expansion of hPSCs as aggregates and their integrated differentiation for production of homogenous hPSC-CM aggregates. hPSCs were initially expanded in a stirred bioreactor as size-controlled homogenous aggregates. Then, aggregates with different sizes directly induced to differentiate into CMs by a simple stepwise protocol optimized for suspension culture under carrier-free conditions without the use of additional extracellular matrix or high cost recombinant proteins or peptides. Time course analysis of beating in various sized

spheroids (90±30, 175±25, and 250±32 µm) showed that cardiac induction occurred in 5-day spheroids (175±25 µm in diameter) at an earlier time and with greater efficiency than in 7-day spheroids (250±32 µm), while 3-day spheroids disrupted after induction. These results support previous reports that recognized the size of hESC aggregates or EBs as a key parameter that influencing the induction efficiency of cardiac and other lineages[43, 44]. The low diffusion rate of growth factors, chemicals, and nutrients, as well as oxygen gradients inside the cell aggregates might underpin the delayed differentiation observed in 7-day spheroids. On the other hand, treating cell aggregates with CHIR decreased hPSCs aggregate integrity in dynamic culture conditions and increased cell loss after differentiation induction that explain the disruption of 3-day aggregates after induction. Therefore, generation of size-controlled aggregates in hPSCs expansion cultures by controlling hydrodynamic culture conditions and defining the optimum aggregate size for a specific differentiation protocol is crucial to achieve a homogenous, efficient CM differentiation.

In optimized culture conditions, a stirred bioreactor containing hPSC aggregates (80-90 × 10^6 of cells in a 100 ml working volume after 5 days of culture) directly differentiated to cardiomyocytes with optimized differentiation cocktails and strategy. With this approach, approximately 100% of the undifferentiated aggregates generated cardiospheres that showed spontaneous contractility and contained highly enriched CMs (up to 90% $cTNT^+$ and MHC^+ cells) with high functionality *in vitro*. This high purity and functionality could facilitate the development of an integrated, cost-effective purification system to generate large-scale hPSC-CMs, required for further applications.

Although, our proposed strategy provides a robust, cost-effective and universal platform for large-scale generation of hPSC-CMs, we believe that the efficacy of the current proposed

strategy can be further improved by optimization of bioprocess parameters in fully controlled conditions. These parameters include oxygen tension, hydrodynamic culture conditions, feeding and media refreshment strategy, and the development of innovative SM delivery technologies for increasing differentiation efficacy and homogeneity, while minimizing hPSCs cell loss after differentiation induction. More recently, it has been demonstrated that optimizing bioprocess parameters including oxygen tension (hypoxia) and bioreactor hydrodynamics can boost mouse iPSC differentiation towards CMs [45]. However, these finding should also be validated for differentiation of hPSC cell lines.

While our study was in preparation, Kempf *et al*. reported the development of a similar small molecule CM differentiation and culturing protocol applied to three hESC and hiPSC lines as aggregates in 100 ml stirred bioreactors [46]. Their study applied sequential CHIR99021 (7.5 µM compared to 12 µM in our study) and IWP2 (5 µM) treatments separated by 2 days (rather than the 1 day used in our study), and did not include the TGFβ signaling inhibitor SB431542 or SHH pathway inducer Pur. Interestingly, after scaling up the optimized differentiation protocol from 12 well plates to Erlenmeyer flasks, and finally the stirred bioreactor, hPSCs expanded in batch stirred bioreactor culture as aggregates with 283±9.6 µM average diameter failed to produce hPSC-CMs in differentiation phase. However, hPSCs aggregates with larger diameter (470-530 µM for different cell lines) that were produced in a continuous culture using a cyclic perfusion feeding strategy, generated contracting hPSC-CMs after 6-10 days of differentiation induction. Here, we have demonstrated that functional and beating hPSC-CMs can be generated in simplified batch and single unit operation from 5 days aggregates with 175±25 µm average diameter after seven days of induction. This appears to be universally applicable as it was reproducible for 9 hESCs and hPSCs cell lines. The simplified and batch operation mode of our

protocol may offer an advantage over the strategy of continuous culture using perfusion feeding which suffers from higher cost, complexity of process control in large scale culture, as well as considerable cell loss during perfusion (~25%). In addition, hPSC-CMs that are produced in our culture system have a smaller average diameter (150-200 μM) compared to hPSC-CMs generated from the perfusion feeding strategy (about 1 mm). The smaller and more homogenous cardiosphere sizes will facilitate their downstream processing (e.g. enzymatic dissociation and purification) and future use for cell therapy and drug discovery applications.

With respect to yield and universality, the continuous culture mode resulted in hPSC-CMs yields similar although lower than ours for the hESC line (67-81% MHC^+ cells in n=3 trials after 10 days of induced differentiation) and apparently more variable with the hiPSC lines (range 27-83% MHC^+ cells in n=3 trials). Further work is required to dissect the practical basis for these differences.

Conclusion

hPSCs likely have the potential to differentiate into all cell types of the body and constitute an extremely attractive tool for generation of cells for cell therapy and other biomedical applications once the safety and scale-up issues have been overcome. An integrated, robust bioprocess for mass production of hPSC-CMs will pave the way for commercializing and clinical application.

Up to now, there are three published reports [47-49] and 11 approved clinical trials involving hPSC-based therapies registered on the US National Institutes of Health clinical trials website (www.clinicaltrials.gov), with nine for ocular indications (eight from hESCs, one from hiPSCs [50]), one for diabetes and one for severe heart failure. Although hPSC-CMs still seems to be some way from clinical application, their large scale production will also provide an opportunity for

the development and refinement of screening platforms for drug toxicity and modeling of diseases including congenital heart disease, long-QT syndromes and Tymothy syndrome (for review see [51, 52]). The availability of massive numbers of human CMs will further provide broad scope for cardiac tissue engineering, *in vitro* organ development, molecular cardiovascular research, and the development of safer, more effective drugs for cardiovascular therapies using high throughput technologies.

The protocol described in this study allows production of hPSC-CMs in a simplified and robust process from different hPSCs lines. It offers advantages over currently demonstrated suspension protocols, which are variously limited in scalability, complexity, affordability, efficacy or CMs functionality. Billions of hPSCs-CMs can be produced using the proposed scale-out and scale-up strategies using hiPSCs or hESCs as starting cells for production and purification (Figure 7). Optimizing key bioprocessing parameters is now an imperative that should be implemented.

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Figure legends

Figure 1. Experimental design and optimization of chemically induced human pluripotent stem cells (hPSCs) differentiation to cardiomyocytes (CMs) in a static suspension system. We used the human embryonic stem cell line (hESC, RH5) in this step. (A) 5-day spheroids formed in suspension culture (spinner flasks) were transferred to low-attachment dishes that contained differentiation medium. For mesoderm induction, hPSCs spheroids were treated for one day with 12 μM CHIR. Then, CHIR was removed and cells cultured for one more day in differentiation media without SMs. At the end of this stage precardiac mesoderm was formed. To obtain cardiac progenitors, we treated spheroids with IWP2, SB431542, and purmorphamine (Pur, 5 μM each) for two days, after which media were renewed every 2-3 days until the end of the study (day 30). (B) The effect of spheroid size on CM differentiation. First beating in 5-day and 7-day spheroids was observed at days 7 and 10. All of the 5-day spheroids were beating by day 10, while the percentage of beating spheroids increased slowly and reached 100% by day 10 in 7-day spheroids. (C) Evaluation of cardiac differentiation by counting the number of beating spheroids (%) and flow cytometry analysis of α-MHC-expressing cells (%) indicated that increasing CHIR concentration to 12 μM resulted in increased CMs differentiation. (D) Flow cytometry analysis of α-MHC-positive cells (%) and calculating the number of beating spheroids (%) showed that the combination of IWP2, SB431542 and Pur led to the most efficient CM differentiation in a static suspension system. All data are presented as SD (n=3).

Figure 2. Reproducibility and CM differentiation pattern of hPSCs in a static suspension system. (A) 5-day spheroids of different hESC and hiPSC lines followed a similar differentiation pattern in which all cell lines showed a beating initiation point at day 7 and a plateau with nearly

100% beating spheroids at day 10. Error bars represent SD (n=3). (B) Flow cytometry analysis of $cTnT^+$ cells indicated that the efficiency of CM generation from hESCs (RH5) and hiPSCs (618-3, 913-5 and 616-1) reached 90% by day 15 of differentiation. Error bars represent SD (n=3).

Figure 3. Experimental design of chemically-induced hESC differentiation to CMs in a dynamic system. (A) 5-day spheroids formed in suspension culture (spinner flasks) were transferred to a new spinner flask that contained differentiation medium (Figure 1A). Rock inhibitor was used for one day and PVA was used for the first two days. *: Represents the first beating at day 7 of differentiation. (B) Time course of development of spontaneously beating spheroids. The 5-day RH5 hESC spheroids began beating at seven days after induction of differentiation. The maximum number of beating spheroids (>90%) was observed at day 10. Error bars represent SD (n=3). (C) Immunostaining of hESC derived-beating spheroids sectioned at day 30 for CM-specific markers. (D) Dissociated beating spheroids were stained for CM specific transcription factor (NKX2-5) and structural proteins (MLC2a and cTNT).

Figure 4. Expression patterns of pluripotency and cardiac lineage-related markers in a dynamic suspension system. (A) Differentially expressed genes could be grouped into three clusters. Cluster 1 included pluripotency genes (*OCT4* and *NANOG*) and a mesodermal marker (*T*) and showed a reduction in gene expression over the time course of CM differentiation. The genes collected in cluster 2 represented upregulation after differentiation induction followed by downregulation over the differentiation process. Cluster 3 included genes that encode CM specific transcription factors and structural proteins. These genes showed sustained upregulation throughout CM differentiation. (B) Principal components analysis (PCA) of expression values in

the dynamic suspension system. In the left panel, each point represents a replicate sample and the colors show the days after differentiation. The right panel represents the location of each gene in the same PCA plot. This analysis shows the expression of pluripotency and CM related genes matched the days after differentiation induction. (C) Quantitative data on dissociated cells from RH5 hESC spheroid showing cardiac lineage markers at different time points during differentiation in the dynamic system. Error bars represent SD (n=3).

Figure 5. Extracellular field potentials recorded from beating spheroids using microelectrode array. (A) Microelectrode array recording showed typical electrical properties of hESC spheroid–derived CMs. (B) Drug response of line RH5-derived CMs showed increased chronotropy when challenged with isoproterenol (Iso), a β-adrenergic agonist.

Figure 6. Action potentials recorded from single beating hPSC-derived CMs.

Action potentials (APs) of single beating hESC-derived CMs; (A) Ventricle-like APs, (B) Atrial-like APs and (C) Nodal-like APs. The classification of different cardiac cell types was based on the morphology of APs (left panels) and AP parameters. All AP recordings were performed at room temperature.

Figure 7. An integrated, robust bioprocessing platform for large-scale production of hPSC-CMs. Production of large-scale hPSCs-CMs require hESCs (derived from human blastocysts) and hiPSCs (generated from patient fibroblasts) as starting material. Expansion of these cell lines as single-cell inoculated suspension cultures in stirred suspension bioreactors using scale-up and scale-out strategies resulted in a few billion to billions of undifferentiated hPSCs. The hPSCs could be easily differentiated in an integrated single unit operation to hPSC-

CMs during 10-15 days**.** Purification of hPSC-CMs could be further achieved by culturing produced cells within lactate-enriched medium. The resultant cells offer tremendous advantages for developing different applications that require large numbers of cells such as high throughput screening and drug discovery, *in vitro* organ development and cardiac tissue engineering.

Supplementary Figure Legend

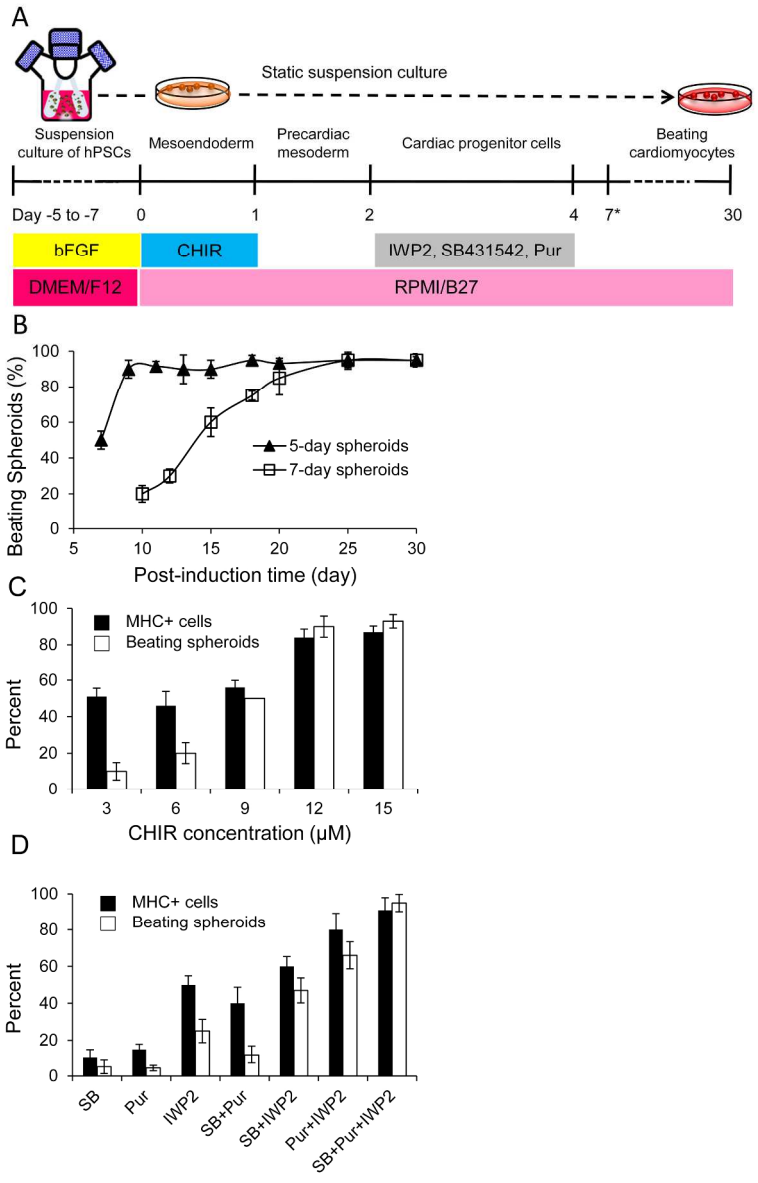
Supplementary Figure 1. Gene expression pattern of cardiogenic genes in a static suspension system. Human embryonic stem cells (hESCs, RH5 line) were differentiated to cardiomyocytes (CMs) in a static suspension system. Temporal gene expression analysis was performed by quantitative RT–PCR at different time points out to 30 days. Error bars represent SD (n=3).

Supplementary Figure 2. cTNT protein expression pattern in a static suspension system. 5-day spheroids derived from RH5 hESCs were differentiated to CMs and analyzed at day 15. The majority of individualized cells (up to 90%) were CMs ($cTnT^+$) while endothelial cells (vWF^+) and vascular smooth muscle cells (αSMA^+) comprised a small proportion of differentiated cells.

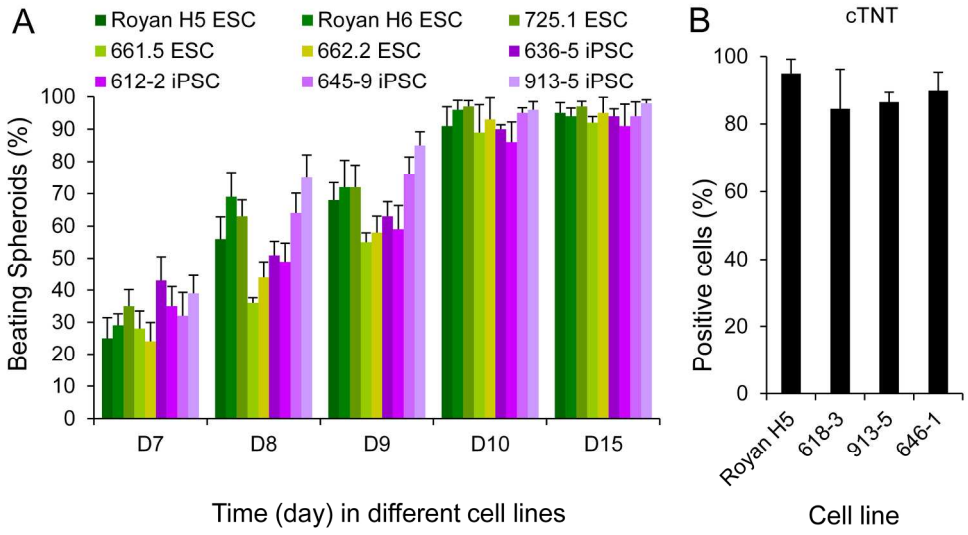
Supplementary Figure 3. Gene expression analysis during CM differentiation in a dynamic suspension system. Quantitative RT-PCR analysis of differentiation of hESCs (RH5 line) to CMs indicated that the expression of pluripotency genes (*OCT4* and *NANOG*) decreased after differentiation induction, whereas cardiac lineage related genes increased in expression. Target genes were normalized to the reference gene *GAPDH.* The relative expression was calculated by dividing the normalized target gene expression of treated samples with that of the undifferentiated state (day 0). All data are shown as log 2 linear plots. Error bars represent SD (n=3).

Supplementary Figure 4. Electrophysiological properties of single beating hiPSC-derived CMs at day 30 of differentiation using the patch clamp technique. Spontaneous APs recorded

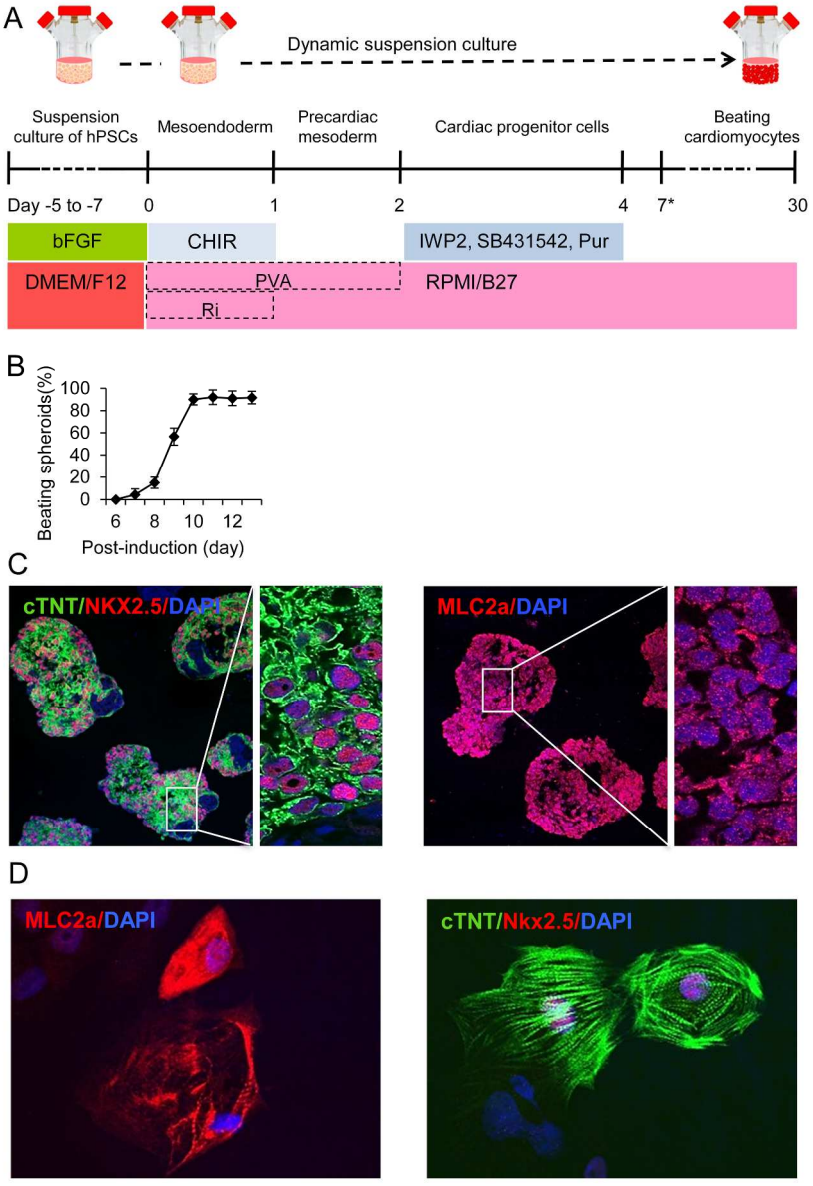
from VC913-5 spheroid–derived CMs. (B) Distribution of (mean ± SEM) AP duration at 90% (APD90) repolarization and (C) beating frequency for n=8 separate cells. (D) Example of APs recorded during addition of quinidine, 100 μM. Expanded traces in panels (a)-(d) show control, early quinidine, late quinidine and recovery after washout. All AP recordings performed at 37°C.



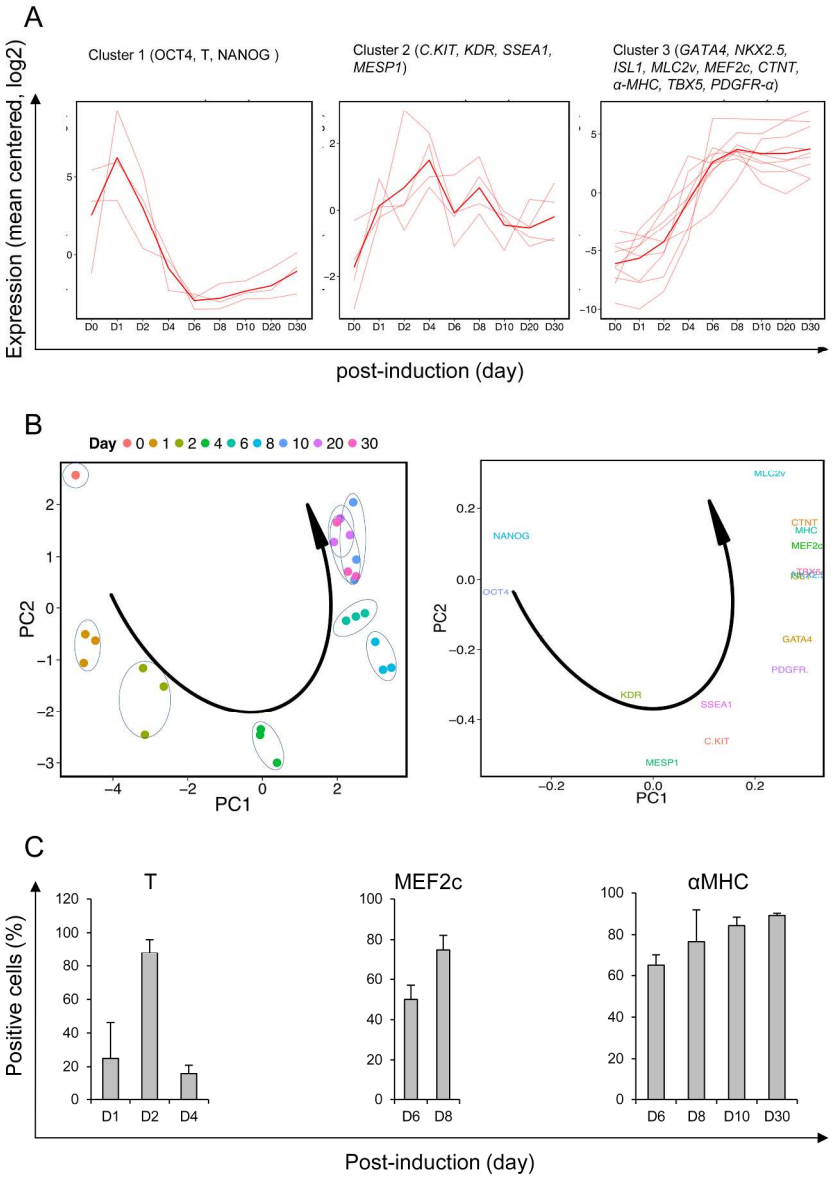
193x297mm (300 x 300 DPI)



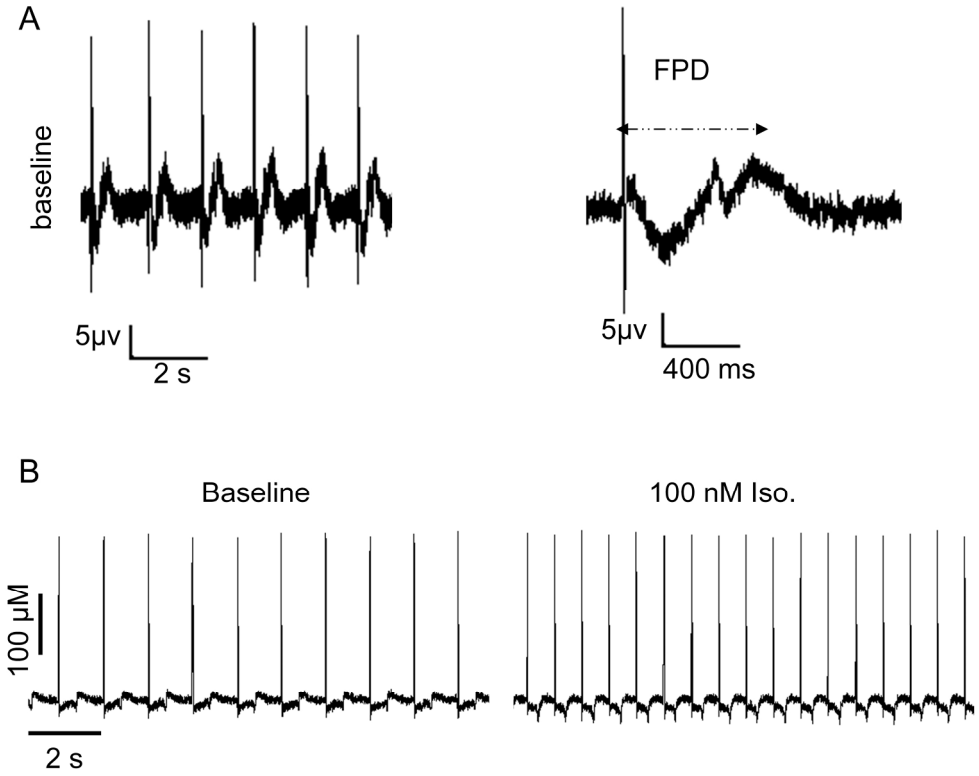
209x117mm (300 x 300 DPI)



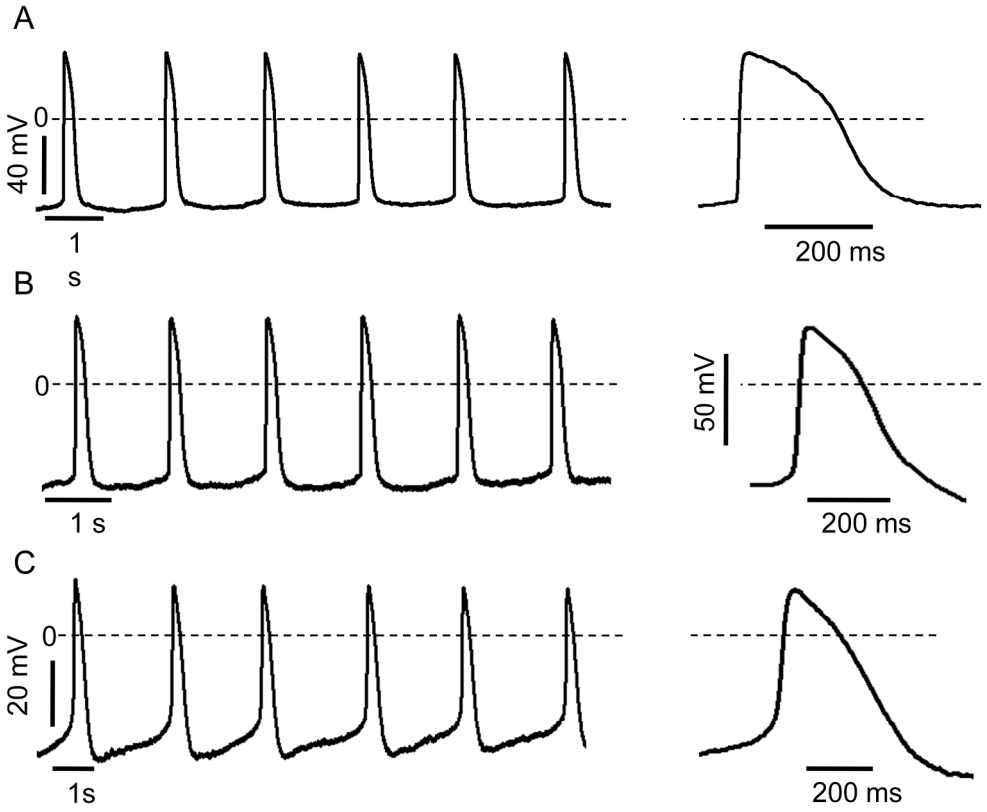
209x297mm (300 x 300 DPI)



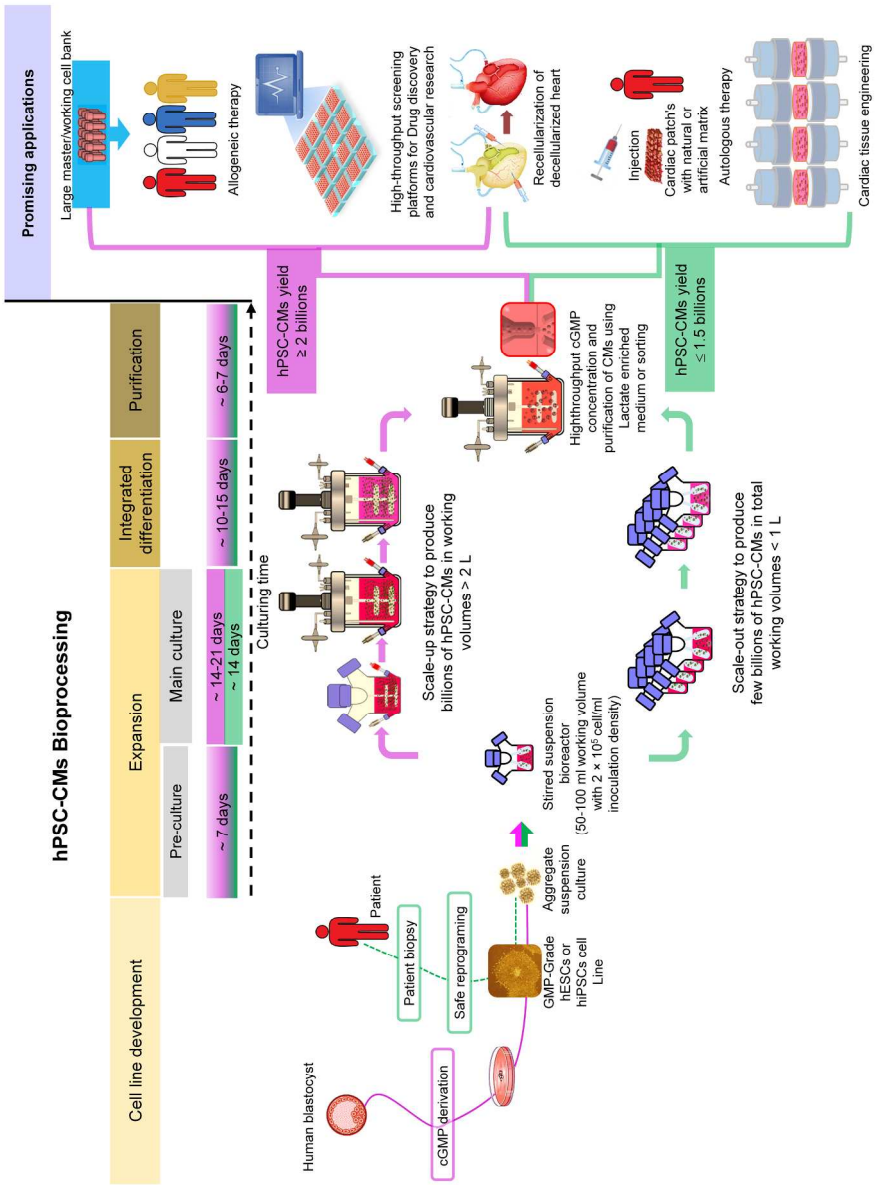
209x297mm (300 x 300 DPI)



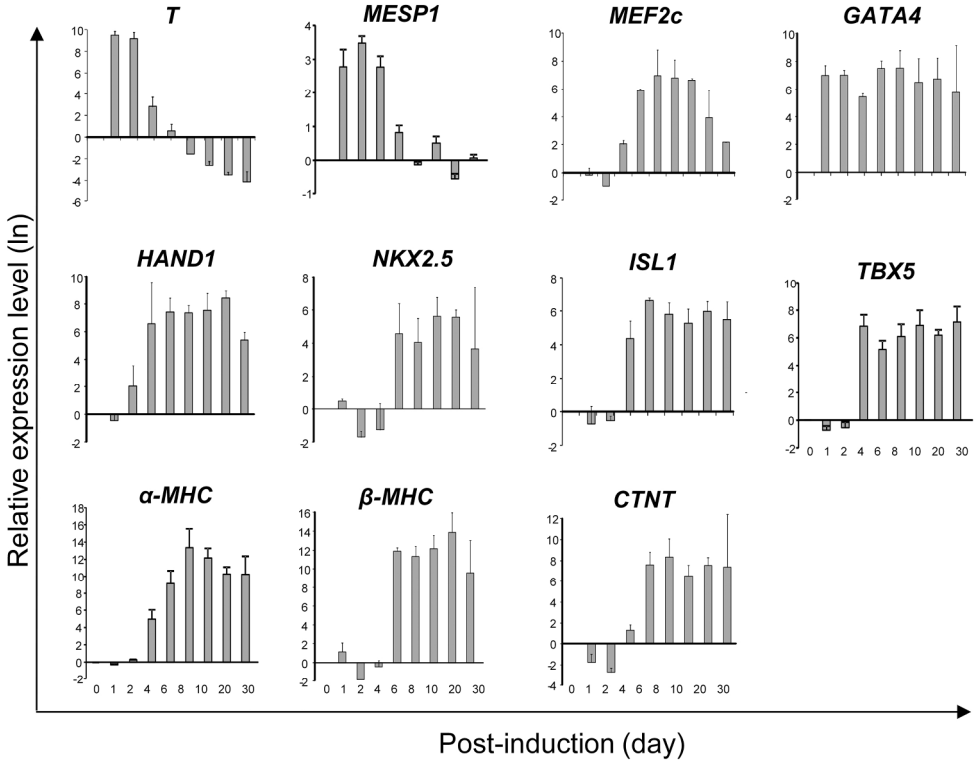
209x168mm (300 x 300 DPI)



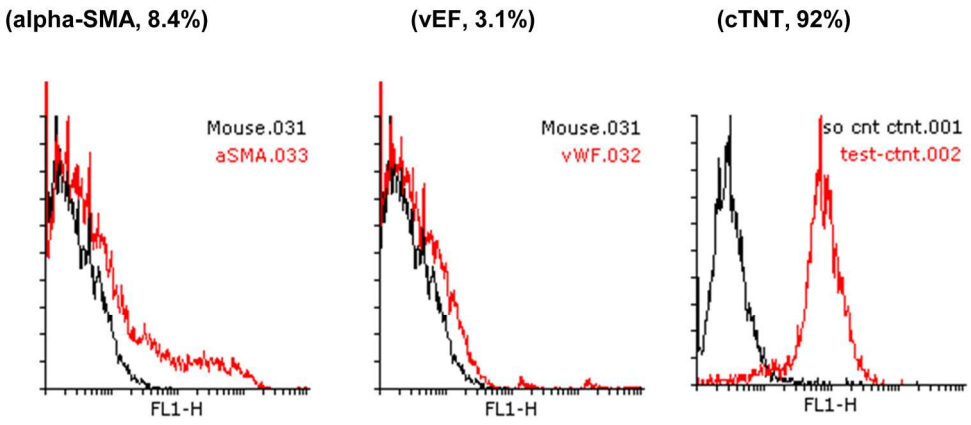
209x175mm (300 x 300 DPI)



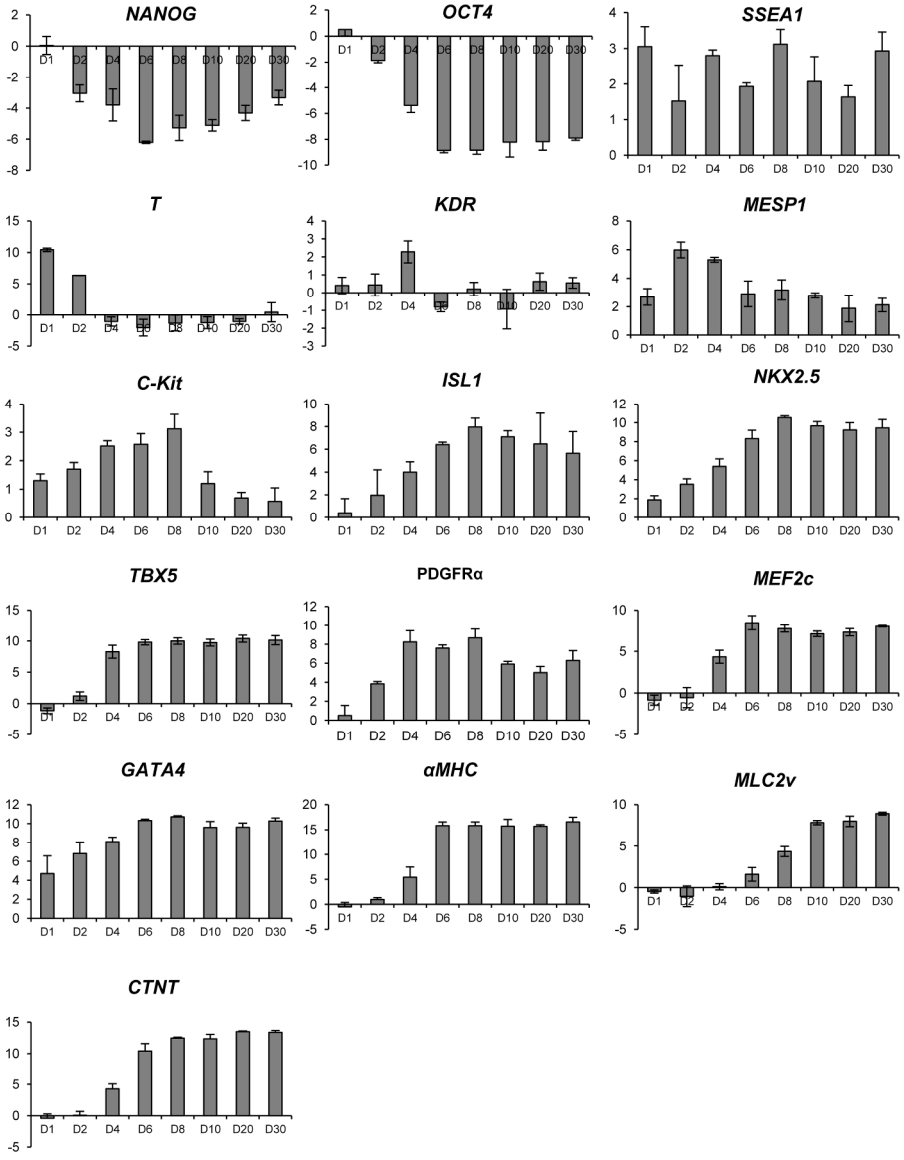
209x284mm (300 x 300 DPI)



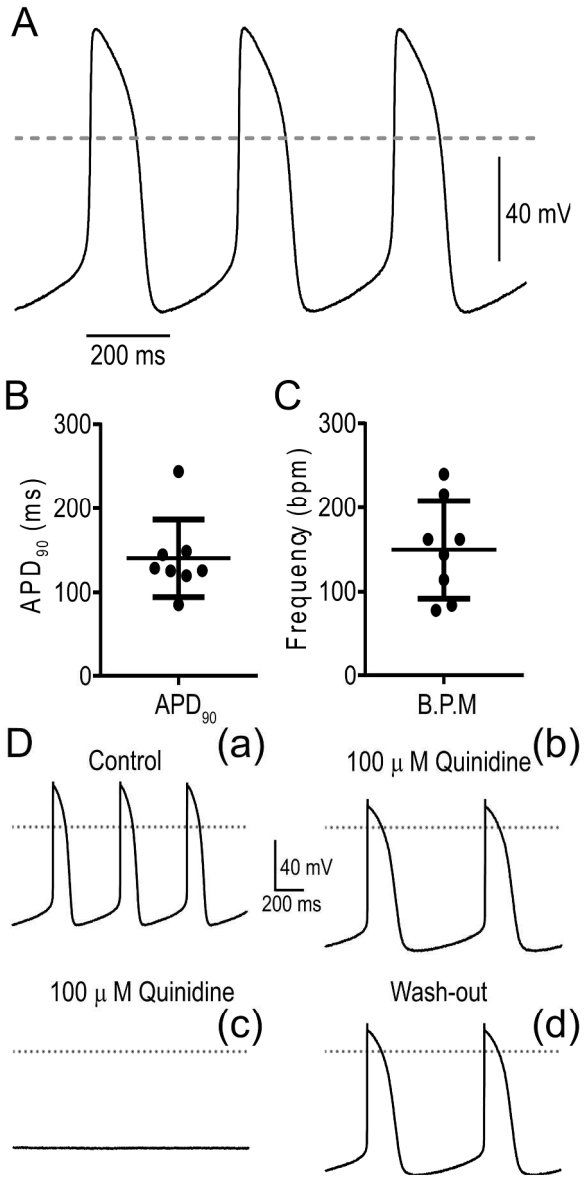
209x164mm (300 x 300 DPI)



209x89mm (300 x 300 DPI)



209x271mm (300 x 300 DPI)



152x297mm (300 x 300 DPI)

Supplementary Table 1: Primers used for real-time PCR (RT-PCR).

Gene name	Sequence (5'-3')
CTNT	F: ATGATGCATTTTGGGGGTTA
	R: CAGCACCTTCCTCCTCTCAG
α-MHC	F: ATTGCTGAAACCGAGAATGG
	R: CGCTCCTTGAGGTTGAAAAG
T	F: AATTGGTCCAGCCTTGGAAT
	R: CGTTGCTCACAGACCACA
c-KIT	F: ATTGTTCTGTGGACCAGGAG
	R: GGTTGTTGTGACATTTGCTG
KDR	F: AAGTATGTGACCCCAAATTCC
	R: AGAACAACACTTGAAAATCTG
MESP1	F: GACGTGCTGGCTCTGTTG
	R: TGTCACTTGGGCTCCTCAG
CXCR4	F: CACCGCATCTGGAGAACCA
	R: GCCCATTTCCTCGGTGTAGTT
PDGFR-α	F: ACAGGTTGGTGTGGGTTCAT
	R: CTGCATCTTCCAAAGCATCA
ISL1	F: TACAAAGTTACCAGCCACC
	R: GGAAGTTGAGAGGACATTGA
NKX2-5	F: TCTATCCACGTGCCTACAG
	R: CCTCTGTCTTCTCCAGCTC
GATA4	F: CCTGTCATCTCACTACGG
	R: GCTGTTCCAAGAGTCCTG
TBX5	F: CGATCACAGATACAAATTCGC
	R: CAGGTGGTTGTTGGTGAGC
MEF2C	F: TCCGAGTTCTTATTCCACC
	R: ATCCTCCCATTCCTTGTC
Mlc2v	F: CTTGGGCGAGTGAACGT
	R: CTGGTCAACCTCCTCCTTG
OCT4	F: GTTCTTCATTCACTAAGGAAGG
	R: CAAGAGCATCATTGAACTTCAC
NANOG	F: AAAGAATCTTCACCTATGCC
	R: GAAGGAAGAGGAGAGACAGT
GAPDH	F: CTCATTTCCTGGTATGACAACGA
	R: CTTCCTCTTGTGCTCTTGCT
SSEA1	R: GTTGGTGGTAGTAGCGGACC
	F: CTTCAACTGGACGCTCTCCTA

Supplementary Table 2: Antibodies used for immunostaining.

Antibody name	Company	Cat. number
cTNT	Thermo Scientific	MS-295-P1
NKX2-5	Santa Cruz	Sc-14033
MLC2a	Santa Cruz	Sc-66967
Goat anti-rabbit Alexa Fluor 555	Life Technologies	A21429
Goat anti-mouse Alexa Fluor 488	Life Technologies	A21424
T	R&D Systems	AF2085
cTNT	Thermo Scientific	MS-295-P1
NKX2-5	Santa Cruz	Sc-14033
MLC2a	Santa Cruz	Sc-66967
MEF2C	Abcam	ab64644
MYH6	Abcam	Ab15