A Massive Suspension Culture System With Metabolic Purification for Human Pluripotent Stem Cell-Derived Cardiomyocytes

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**Abstract**

Cardiac regenerative therapy with human pluripotent stem cells (hPSCs), such as human embryonic stem cells and induced pluripotent stem cells, has been hampered by the lack of efficient strategies for expanding functional cardiomyocytes (CMs) to clinically relevant numbers. The development of the massive suspension culture system (MSCS) has shed light on this critical issue, although it remains unclear how hPSCs could differentiate into functional CMs using a MSCS. The proliferative rate of differentiating hPSCs in the MSCS was equivalent to that in suspension cultures using nonadherent culture dishes, although the MSCS provided more homogeneous embryoid bodies (EBs), eventually reducing apoptosis. However, pluripotent markers such as Oct3/4 and Tra-1-60 were still expressed in EBs 2 weeks after differentiation, even in the MSCS. The remaining undifferentiated stem cells in such cultures could retain a strong potential for teratoma formation, which is the worst scenario for clinical applications of hPSC-derived CMs. The metabolic purification of CMs in glucose-depleted and lactate-enriched medium successfully eliminated the residual undifferentiated stem cells, resulting in a refined hPSC-derived CM population. In colony formation assays, no Tra-1-60-positive colonies appeared after purification. The nonpurified CMs in the MSCS produced teratomas at a rate of 60%. However, purified CMs never induced teratomas, and enriched CMs showed proper electrophysiological properties and calcium transients. Overall, the combination of a MSCS and metabolic selection is highly effective and practical approach to enrich and enrich massive numbers of functional CMs and provides an essential technique for cardiac regenerative therapy with hPSC-derived CMs.

**Introduction**

Heart failure (HF) is a notorious disease in developed countries, despite significant medical advances in previous decades. Currently, cardiac transplant is a last resort to treat patients with refractory HF [1]. However, a shortage of transplantable hearts is an unresolved problem. To prevent graft rejection, the transplant recipients require immunosuppressive therapy, which has been linked to toxic side effects, malignancy, and infection. The median survival rate of heart transplant patients is approximately 10 years owing to coronary allograft vasculopathy [2]. An alternative radical treatment is thus imperative for patients with severe HF. Regenerative medicine is one such approach to achieve this unmet medical need to regain contractile function with newly generated cardiomyocytes (CMs). Notably, human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) have pluripotency to differentiate into all cell types in vitro and in vivo, including CMs [3–5]. The use of hiPSCs is particularly advantageous, because it avoids the ethical issues and post-transplant immunosuppressive therapy associated with the use of hESCs [6]. In general, the rate at which hPSCs differentiate into CMs varies among cell lines [7]. Therefore, devising an efficient strategy to gain large-scale production of purified CMs will be the key to success in cardiac regenerative therapy. A major hurdle to overcome is the lack of an appropriate method to cost-effectively produce a sufficient number of CMs for clinical therapy. To overcome this fundamental problem, a massive suspension culture system (MSCS) using a spinner flask or a bioreactor was proposed. The suspension culture of undifferentiated murine and human pluripotent stem cells (hPSCs) was effective in maintaining large cell numbers in the pluripotent state [8,
The MSCS was also used for cardiac differentiation, resulting in the efficient generation of CMs from murine and hESCs [10, 11]. The morphology, molecular markers, and electrophysiological properties were compatible with those of functional CMs [12, 13]. However, although hPSCs effectively differentiate into CMs, they must be purified using an efficient selection system, because hPSC-derived embryoid bodies (EBs) grown in a MSCS also contain various types of noncardiac cells [14–16]. Teratoma formation from residual undifferentiated stem cells is one of the most concerning potential risks of hPSC-derived CM transplantation [17]. Several selection systems have been reported to purify PSC-derived CMs, and most have used fluorescent activated cell sorting (FACS) or drug selection after genetic modification [18–21]. We have previously demonstrated distinct metabolic differences between CMs and noncardiac proliferative cells, including PSCs, using a combination of transcriptome and metabolome analyses [22]. In brief, we found that hPSCs and other proliferative cells depend on glycolysis to produce ATP and biomass such as amino acids and nucleotides, and CMs can effectively use lactate, as well as glucose, via oxidative phosphorylation to generate energy, owing to their well-developed mitochondria. Considering these metabolic differences, we created a glucose-depleted and lactate-supplemented medium that suppressed noncardiac cells, including undifferentiated stem cells, and enriched a population of CMs. This method provided a novel system for purification of CMs from hPSC-derived mixed cell populations [22]. However, it remains unclear whether such a metabolic selection system is effective for preparing the large number of hPSC-derived CMs required for clinical application, free from contamination with undifferentiated stem cells.

In the present study, we propose a practical culture system for generating substantial numbers of refined CMs by combining a MSCS with a metabolic selection medium; thus, taking a step toward realizing the potential of cardiac regenerative medicine using hPSC-derived CMs.

**Materials and Methods**

**Animals**

NOD-SCID mice (8 weeks old, male) were purchased from Japan CLEA (Tokyo, Japan). The Animal Care and Use Committees of Keio University approved all experimental procedures and protocols, which conformed to the NIH Guide for the Care and Use of Laboratory Animals.

**Reagents**

The mouse monoclonal antibodies for α-actin and connexin 43 were purchased from Sigma-Aldrich (St. Louis, MO, http://www.sigmaaldrich.com). The mouse monoclonal antibodies for tropo- nin T (cardiac) and Tra-1-60 were purchased from Thermo Scientific (Wilmington, DE, http://www.nanodrop.com) and Millipore (Billerica, MA, http://www.millipore.com), respectively. The goat polyclonal antibody for Nkx2-5 (N-19) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, http://www.scbt.com). Alexa Fluor 488 and 546 anti-mouse IgG antibody and anti-goat IgG antibody and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen (Carlsbad, CA, http://www.invitrogen.com). Fibronectin was purchased from Sigma-Aldrich.

**Maintenance of Human iPSCs and ESCs**

The hiPSC line (253G4) was obtained from the Center for iPSC Research and Application, Kyoto University (Kyoto, Japan). The hESC line (Khes-2) was obtained from the Department of Development and Differentiation, Institute for Frontier Medical Sciences, Kyoto University. We maintained hiPSCs on SNL feeder cells in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 Ham 1:1 (DMEM-F12; Invitrogen) supplemented with 20% knockout serum replacement (Invitrogen), 0.1 mM minimal essential medium (MEM) nonessential amino acids solution (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), and 5 ng/ml basic fibroblast growth factor (Wako Pure Chemical, Osaka, Japan, http://www.wako-chem.co.jp/english). Karyotypes of hiPSCs were analyzed by Nihon Gene Research Laboratories, Inc. (Sendai, Japan, http://www.ngri-japan.com).

**Cardiac Differentiation Using Spinner Flasks**

To form EBs, we cultured enzymatically detached human PSCs (approximately 5 × 10⁶ cells) with 100 ml of mTeSR1 (StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) supplemented with 5 nM bone morphogenetic protein 4 (BMP4) (R&D Systems Inc., Minneapolis, MN, http://www.rndsystems.com) and 10 μM Y27632 in 125-ml spinner flasks (Corning Inc., Corning, NY, http://www.corning.com). Two days later, the medium was changed to StemPro-34 (Invitrogen) supplemented with 50 mg/l ascorbic acid and 2 mM GlutaMAX (Invitrogen). Human BMP4, activin A, and a small-molecule Wnt inhibitor, IWR-1, were added at the indicated points and concentrations (Fig. 1B). The agitation rate was 40–100 rpm during differentiation. EBs were used for purification experiments between days 18 and 20.

**Purification of Human PSC-Derived CMs**

The human PSC-derived EBs at differentiation day 18–20 in the spinner flasks were extensively washed with, and exposed to, the glucose-free DMEM (no glucose, no pyruvate with L-glutamine; Invitrogen) supplemented with 4 mM L-lactic acid (Wako Pure Chemical) for 7 days [22]. The media were changed every 2 or 3 days to eliminate dead cells by rapid flushing. After metabolic selection in the spinner flasks, the contracting CM aggregates were collected and attached onto fibronectin-coated dishes with α-MEM plus 5% fetal bovine serum or StemPro-34 for 2 or 3 days. The attached CM aggregates were then exposed to the glucose-free DMEM with 4 mM L-lactic acid to obtain more purified CMs. A 1-M l-lactic acid stock solution was prepared by diluting 10 M lactate (Wako Pure Chemical) with sterile 1 M HEPES (Invitrogen). The purified CMs were finally collected by rapid flushing.

**Immunofluorescence**

We fixed the cells with 4% paraformaldehyde for 20 minutes. Subsequently, the cells were permeabilized with 0.1% Triton X-100 (Wako Pure Chemical) at room temperature for 10 minutes and then incubated with the primary antibody at 4°C overnight. The cells were then washed with phosphate-buffered saline (PBS) containing 0.1% Tween-20 four times before incubation with the secondary antibodies at room temperature for 2 hours. After nuclear staining with DAPI,
the stained cells were detected using fluorescence microscopy (Axio Observer; Carl Zeiss, Jena, Germany, http://www.zeiss.com).

**Colony Formation Assay**

Nonpurified and purified hiPSC (253G4)-derived cells \(2.0 \times 10^5\) were completely dissociated and cultured on SNL feeder cells under PSC maintenance culture conditions with 10 \(\mu\)M Y27632 for 4 days. Then, immunofluorescence staining for Tra-1-60 was performed, and the colonies were counted.

**Assessment of Apoptosis by Annexin V and Propidium Iodide Staining**

After complete dissociation of hiPSC (253G4)-derived EBs at day 10, the cells were stained with Alexa Fluor 488 annexin V and propidium iodide (PI), following the manufacturer’s instruction (Invitrogen). In brief, after the cells were treated with Alexa Fluor 488 annexin V and PI at room temperature for 15 minutes, they were resuspended in 400 \(\mu\)l of annexin-binding buffer and kept on ice. These cells were analyzed using FACS (Gallios; Beckman Coulter, Fullerton, CA, http://www.beckmancoulter.com).
FACS Analysis Using Sarcomeric α-Antin Actin Antibody

Purified CMs were completely dissociated by 0.25% trypsin-EDTA and then fixed with 4% paraformaldehyde for 10 minutes. Subsequently, the cells were permeabilized with 0.1% Triton X-100 at room temperature for 10 minutes and then incubated with the anti-α-actinin antibody (Sigma-Aldrich) at 4°C overnight. The cells were washed with PBS containing 0.1% Tween-20 before incubation with the Alexa Fluor 488 donkey anti-mouse IgG secondary antibody (Invitrogen) at room temperature for 2 hours. These cells were analyzed by FACS (Gallios; Beckman Coulter).

Reverse Transcription-Polymerase Chain Reaction and Real-Time Polymerase Chain Reaction

Total RNA was extracted using an RNasy Mini Kit (Qiagen, Hilden, Germany, http://www.qiagen.com), and the cDNA was synthesized using the Superscript First-Strand Synthesis System (Invitrogen). Reverse transcription-polymerase chain reaction (RT-PCR) was performed, as described previously [23]. For quantitative analysis, cDNA was used as the template in a TaqMan real-time PCR assay using the LightCycler 96 System sequence detection system (Roche, Indianapolis, IN, http://www.roche.com) according to the manufacturer’s instructions. The data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers and TaqMan probe for human POUSF1 (OCT3/4), ACTC1, NKX2.5, TNNT2, MYL2, MYL4, and GAPDH were Hs01895061_m1, Hs00606316_m1, Hs00231763_m1, Hs00165960_m1, Hs00166405_m1, Hs00267321_m1, and Hs02758991_g1, respectively. The primers used in the RT-PCR are listed in Table 1.

| Gene       | Direction | Sequence (5’ to 3’) | Size (bp) |
|------------|-----------|---------------------|-----------|
| GAPDH      | Forward   | AACACAGTTCCATGGCCTACAC | 556       |
|            | Reverse   | TCCACCACTTGGTGCTGA   |           |
| POUSF1     | Forward   | CCTGGGGGTTCTATTTGGGA | 530       |
|            | Reverse   | TTGAATGCAATGGAGAGCC  |           |
| BRACHYURY T| Forward   | CAACCTCAGTGAAGGTGAA  | 100       |
|            | Reverse   | ACAAAAATCGGTTGTCGCAA|           |
| MESP1      | Forward   | CGCTCTGAGGGCAAGAAG   | 101       |
|            | Reverse   | GCAATGTGCAAGGAACAC   |           |
| TNNT2      | Forward   | GAAGAAAGATCTGGTGAATT | 208       |
|            | Reverse   | GGCATTTGAGCATCTCTG    |           |
| ACTC1      | Forward   | GCGTGCAGTCACACTG     | 580       |
|            | Reverse   | ATGCTAAGGTCAGGCCCC    |           |
| NKX2-5     | Forward   | CCCCCTCAGTCAAACAGATC  | 228       |
|            | Reverse   | AAAGCAGAAGGCAACTCT    |           |

Abbreviation: RT-PCR, reverse transcription polymerase chain reaction.

Teratoma Formation

To verify the elimination of undifferentiated stem cells with the potential to form teratomas, we transplanted 2.0 × 10^5 purified hiPSCs (253G4)-derived CMs, nonpurified hiPSCs (253G4)-derived cells, and undifferentiated hiPSCs (253G4) subcutaneously into immunocompromised NOD-SCID mice. The cells were suspended in media with growth factor-reduced Matrigel (BD Biosciences, San Diego, CA, http://www.bdbiosciences.com). Two months after transplantation, the mice were euthanized, and the teratoma incidence was evaluated.

Action Potential Recordings

The whole-cell patch-clamp technique was used to record the action potentials using Axopatch 200B, Digidata 1440A, and pClamp 10.2 software (Molecular Devices, Sunnyvale, CA, http://www.moleculardevices.com). Current-clamp recording were conducted in normal Tyrode’s solution containing 135 mM NaCl, 0.33 mM NaH₂PO₄, 5.4 mM KCl, 1.8 mM CaCl₂, 0.53 mM MgCl₂, 5.5 mM glucose, and 5 mM HEPES (pH 7.4 at 37°C) using the pipette solution: 60 mM KOH, 80 mM KCl, 40 mM aspartate, 5 mM HEPES, 10 mM EGTA, 5 mM Mg-ATP, 5 mM sodium creatinine phosphate, and 0.65 mM CaCl₂ (pH 7.2, adjusted with KOH). Amphotericin B was added to the pipette solution (final concentration 0.3 g/L) to perforate the cell membrane just before use.

Field Potential Recordings Using a Multielectrode Array System

To characterize the functional properties of our purified hiPSCs (253G4)-derived CMs, we performed extracellular recording of field potentials using the multielectrode array (MEA) system.

Calcium Imaging

Purified dissociated hiPSCs (253G4)-derived CMs were seeded and cultured for 4 days. They were then labeled with 2 μM fluo-4 dye (Invitrogen) for 30 minutes at 37°C and washed. Fluo-4-labeled cells were observed and analyzed by confocal laser microscopy (LSM SDUO; Carl Zeiss).

Statistical Analysis

Statistical significance was evaluated using a Student’s t test. The values are presented as mean ± SD or SE; p < .05 was considered significant.

RESULTS

Growth of Cardiac Embryoid Bodies in a Massive Suspension Culture System

To check the differentiation potential and cell growth of hPSC-derived CMs in a massive suspension culture system (MSCS), hiPSCs (253G4) and hESCs (KhES-2) were differentiated into CMs in 125-ml spinner flasks in a 37°C incubator and compared with those cultured under a standard suspension culture in non-adherent culture dishes (Fig. 1A). The karyotype of hiPSCs was normal in the undifferentiated state (supplemental online Fig. 1A). The appropriate rotation speed of a spinner flask was evaluated to keep EBs in suspension, and 40–100 revolutions per minute was the most suitable (data not shown). The suspension culture protocol for cardiac differentiation is shown in Figure 1B. In brief, hPSCs were differentiated into mesodermal lineage cells with BMP4, activin A, and ascorbic acid [7, 26]. Subsequently,
the mesodermal cells were differentiated into CMs with Wnt inhibitor, IWR-1 [27]. The proliferative rate and size of differentiating hiPSCs were compared with cells in nonadherent culture dishes. The EBs grew well in both spinner flasks and dishes, although their rate of proliferation was not significantly greater in a spinner flask than on the dishes (Fig. 1C). The EBs in dishes aggregated and thus were larger than the EBs on the dishes (Fig. 1D, 1E). Intriguingly, the MSCS generated more homogeneous and smaller EBs at every measurement point (Fig. 1D, 1F).

Early Apoptotic Cells Were Significantly Increased in Suspension Culture Dishes

To investigate the relevance of a MSCS in cardiac differentiation, the apoptotic rate was analyzed by FACS with the apoptotic markers annexin V and PI [28]. At day 15, as shown in Figure 1D and 1F, EBs in the suspension culture dishes were highly variable in size and shape; thus, the apoptotic rate was checked at day 10. In accordance with the morphological profile, the proportion of early apoptotic cells in the EB cultures was increased at this stage. However, in keeping with the uniform morphology of the MSCS EBs, the proportion of early apoptotic cells did not increase significantly (Fig. 2A). The repetitive data revealed that the early phase of apoptotic cells (annexin V-positive/PI-negative) increased significantly, although the population of necrotic cells (PI-positive) was not different at this stage (Fig. 2B).

Molecular Profiles of Cardiac Differentiation in the MSCS

The molecular profiles of undifferentiated stem cells (253G4), mesodermal cells, and cardiac cells were then investigated by RT-PCR (Fig. 3A). POUSF1 (Oct3/4) expression rapidly decreased, along with differentiation after day 3, although surprisingly, it was still clearly expressed 2 weeks later. The peak of endomesodermal marker, T (brachyury), expression was day 3, at which stage the earliest mesocardiac marker, MESP1, was also detected. NKX2-5, ACTC1 (α-actinin), and TNNT2 (cardiac troponin T) were expressed in succession. The cardiac differentiation markers were also checked by immunocytochemistry, showing positive staining for Nkx2-5, troponin T, and α-actinin, although these markers were only sparsely detected in the CMs (Fig. 3B).

We then verified the expression of another pluripotent marker, Tra-1-60, to confirm the survival of undifferentiated stem cells and found by immunocytochemistry that some

Figure 2. Early apoptotic cells increased in suspension culture dishes. (A): Representative early-phase apoptotic cell population, analyzed using fluorescent-activated cell sorting, in suspension culture dishes and the massive suspension culture system (MSCS). (B): Fewer apoptotic cells were detected in cardiac embryoid bodies from the MSCS than in those cultured in low-attachment cell culture dishes (MSCS, n = 5; dish, n = 4). *, p < .05. Data are shown as mean ± SD. Abbreviations: NS, not significant; PI, propidium iodide.
Tra-1-60-positive stem cells still existed 2 weeks after differentia-
tion (Fig. 3B).

Metabolic Purification With Glucose-Depleted and
Lactate-Supplemented Medium

POU5F1 mRNA was clearly expressed in fully differentiated CMs in
the MSCS. Tra-1-60-positive undifferentiated stem cells were also
detected by immunocytochemistry. Thus, the cardiac differenti-
ation protocol for the MSCS was modified by the addition of a met-
abolic purification system that started at days 18–20 after
mitochondrial maturation (Fig. 4A). Cardiac EBs were smaller af-
ter purification owing to the death of noncardiac proliferative
cells (Fig. 4B; supplemental online Videos 1, 2). The proportion
of α-actinin-positive CMs before metabolic selection was not
significantly higher in the MSCS, but the differentiation effi-
ciency varied widely in the suspension culture dishes (Fig. 4C;
supplemental online Fig. 1B). More than 99% of cells were posi-
tive for α-actinin on FACS after the modified metabolic purifica-
tion (Fig. 4C). Immunocytochemical analysis also confirmed that
almost all purified CMs were α-actinin-positive and were success-
fully enriched (Fig. 4D; supplemental online Fig. 2B). For the pur-
pose of evaluating the potential tumorigenicity of PSC-derived
CMs from an MSCS, colony formation assays were performed
with iPSCs (253G4). Amazingly, the prepurified CMs showed more
than 100 Tra-1-60-positive undifferentiated colonies growing in
2 × 10⁶ cells 4 days after incubation in the PSCs culture conditions.
In contrast, Tra-1-60-positive undifferentiated colonies were not
detected after purification (Fig. 4E). Real-time PCR also confirmed
the complete elimination of undifferentiated stem cells from
the purified CM cultures. Compared with prepurified cardiac EBs,
the expression of POU5F1 was significantly reduced. In contrast,
the cardiac differentiation markers were highly positive against
the prepurified CMs (Fig. 4F). The ventricular marker, myosin light
chain 2v (MYL2), and the atrial marker, myosin light chain 1a
(MYL4), were enriched in purified CMs (Fig. 4G).

Figure 3. The undifferentiated stem cells remained even after complete cardiac differentiation. (A): Molecular markers of cardiac differen-
tiation were checked by reverse transcription-polymerase chain reaction. The pluripotent marker, POU5F1, was still clearly detected 15 days
after cardiac differentiation. (B): The differentiated cells were immunopositive for Nkx2-5, α-actinin, and cardiac troponin-T by immunoflu-
orescence, and Tra-1-60-positive stem cells remained after cardiac differentiation. The density of the cardiomyocytes was obviously low.

Teratoma Formation of hiPSC-Derived CMs
Without Purification

Undifferentiated hPSCs retain the strong potential to induce terato-
mas by differentiating into cells of the three germ layers. This is the
most valuable characteristic of these stem cells and also the one of
most concern for clinical application of hPSCs. To evaluate the po-
tential for teratoma formation by hiPSC-derived CMs from the
MSCS, undifferentiated hiPSCs (253G4) (n = 15), nonpurified CMs
(n = 20), and purified CMs (n = 20) were injected into the subcutane-
ous tissues of NOD-SCID mice. The undifferentiated hiPSCs induced
large teratomas in >90% of the injected mice, and nonpurified CMs
generated much smaller ones in approximately 60% of the mice (Fig.
5A). The size scale of the nonpurified CM-derived teratomas was
approximately 25% of those derived from the hiPSCs (Fig. 5B). No
teratoma formation in any mice was observed after transplantation
of the purified CMs (Fig. 5A, 5B). The teratomas from undifferen-
tiated iPSCs and nonpurified CMs contained components of all three
germ layers (Fig. 5C). These results confirmed the complete extinc-
tion of the undifferentiated stem cell state in the purified CM
populations, thus abrogating the capacity for tumor formation.

Electrophysiological Properties and Calcium Oscillation
of Human iPSC-Derived CMs in the MSCS With
Metabolic Selection

Finally, the functional quality of hiPSC (253G4)-derived CMs
grown in the MSCS was assessed by immunostaining for α-actinin
Figure 4. The metabolic selection of cardiac embryoid bodies (EBs) completely eliminated the undifferentiated stem cells and enriched cardiomyocytes (CMs). (A): Modified protocol for the massive suspension culture system (MSCS) combined with metabolic purification. (B): Representative cardiac EBs at day 20 (prepurification) and day 26 (postpurification) in the MSCS. (C): The proportion of α-actinin-positive CMs was 25.0% before metabolic selection but grew to more than 99.5% in subsequent fluorescent activated cell sorting analyses. (D): Condensed CMs were positive for α-actinin after metabolic selection. (E): Colony formation assays confirmed the complete purge of undifferentiated stem cells for cardiac EBs after metabolic selection. Many Tra-1-60-positive induced pluripotent stem cells grew before metabolic selection, but no Tra-1-60-positive cells were subsequently detected by immunofluorescence (n = 5). *p < .05. (F): The expression of a pluripotent stem cell marker, POU5F1, and cardiac differentiation markers was checked using real-time polymerase chain reaction. After metabolic selection, the expression of POU5F1 was significantly decreased, and the cardiac markers were highly enriched (n = 9). *p < .05. (G): Both atrial and ventricular markers (MYL4 and MYL2) were enriched after purification. *p < .05. Data are shown as mean ± SD. Abbreviations: BMP4, bone morphogenetic protein 4; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco’s modified Eagle’s medium.
and connexin 43. α-Actinin-positive CMs clearly expressed connexin 43 at their edges (Fig. 6A). The action potential of purified CMs was recorded using the whole-cell patch-clamp technique. All three types of action potentials (nodal-, atrial-, and ventricular-like) were clearly detected. One half of the purified CMs showed atrial-like action potential (Fig. 6B–6D). Testing of the electrophysiological function of purified hiPSC-derived CMs from the MSCS using a MEA system revealed a typical electrocardiograph (Fig. 6E). The drug response of purified CMs was also assessed using MEA. The β-stimulant (isoproterenol) increased the contraction frequency in a dose-dependent manner, and the β-blocker (propranolol) decreased it in a similar manner (Fig. 6F). Intracellular Ca\(^{2+}\) signaling was also analyzed using fluo-4 dye to reveal spontaneous and synchronized Ca\(^{2+}\) oscillations in the purified hiPSC-derived CMs (Fig. 6G; supplemental online Video 3).

Figure 5. Teratoma formation assay with hiPSC-derived CMs in the massive suspension culture system (MSCS). (A): No teratoma was found in NOD-SCID mice injected with purified CMs (n = 20). (B): The proportion and size of teratoma formation from CMs in the MSCS were correlated. Note that unpurified CMs in the MSCS still induced teratoma formation at a rate of approximately 60% (n = 20), although the average teratoma size was less than that of those formed from undifferentiated stem cells (n = 15). *= p < .05. (C): Differentiation into the three germ layers in teratoma tissue was verified in both undifferentiated hiPSCs and unpurified hiPSC-derived CMs in the MSCS. Data are shown as mean ± SD. Abbreviations: CMs, cardiomyocytes; hiPSC, human induced pluripotent stem cell; iPSCs, induced pluripotent stem cells.
DISCUSSION

An effective scheme to attain large volumes of purified and functional CMs is a step toward effective regenerative therapies for HF. A suspension culture system was reported to be an efficient technique for maintaining hPSCs in the undifferentiated state and promoting the differentiation of EBs [29]. Thus, although the EBs were expected to sustain some damage from the rotating paddles in the spinner flasks, the MSCS experiments generated homogeneous EBs and fewer apoptotic cells. In contrast, the EB size could not be controlled in the suspension-culture dishes. A variable EB size leads to increased apoptosis and varying efficiency of cardiac differentiation. These results have thus confirmed that the MSCS represents a substantial leap forward in quality control for cardiac development techniques.

The rate at which cells differentiate into CMs depends on the cell line and culture system [7]. The quality varies among hiPSC lines, and some lines tend to differentiate predominantly, or solely, into a specific cell type [30]. In particular, the development of mesodermal lineage cells from PSCs relies on adjacent endodermal lineage cells [31]. Currently, several differentiation methods are available for cardiac differentiation from PSCs [32], and many laboratories use a suspension culture system, including an MSCS, for cardiac differentiation [13, 29, 33]. A comparison

![Figure 6](https://www.StemCellsTM.com)
of these systems with our own indicated that the differentiation and proliferation rate varied from batch to batch, but showed they were all useful for generating large numbers of CMs from hPSCs. Questions remain regarding how much the differentiated CMs grown in an MSCS will retain their potential tumorigenicity. Our PCR data strongly suggest the survival of Oct3/4-positive cells in 2-week-cultured EBs, indicating the presence of residual undifferentiated stem cells, even with the optimized cardiac differentiation protocol. Also, the immunocytochemical data clearly showed Tra-1-60 staining. The colony formation assays confirmed a subset of remaining undifferentiated iPSCs before purification. Additionally, the incidence and size of the teratomas were reduced after differentiation; however, the nonpurified EBs with differentiated CMs still led to a 60% rate of tumor formation. Their growth rate indicated a weak malignant phenotype; however, they still had good potential to differentiate into all three germ layers’ derivatives as complete teratomas. These data strongly emphasize the requirement for excellent purification strategies in large cell culture systems, because the safety of cell transplantation therapies with PSC-derived cells is the single-most critical issue for future clinical success. Metabolic purification with glucose-depleted and lactate-supplemented medium was successful in eliminating undifferentiated stem cells and other non-cardiac cells in small-scale culture systems [22]. In our hands, the MSCS generated approximately $1 \times 10^7$ CMs from $5 \times 10^6$ hiPSCs after purification, thus providing a large number of CMs for clinical applications (1 $\times 10^6$ CMs) when using 10 bottles in the MSCS ($5 \times 10^7$ hiPSCs). If a sequential combination of the MSCS and metabolic selection could be proved successful in additional studies with other PSC lines, sufficient numbers of CMs could be routinely generated to create a reproducible system suitable for clinical application. The subsequent metabolic purification after cardiac differentiation by the MSCS also succeeded in purging the remaining hiPSCs, with our real-time PCR and FACS data confirming a significant reduction in undifferentiated stem cells and enrichment of CMs. Also, importantly, no teratoma formation was induced in the mice by the purified CMs after metabolic selection.

Moreover, CMs purified by metabolic selection showed consistent connexin 43 expression, action potentials, electrophysiological findings, drug responses, and calcium oscillations, indicating that the purified cardiac EBs display electrophysiological functions and calcium transients expected of normal CMs. hESC-derived CMs have previously shown a functional electrophysiological connection with the host heart [34, 35], although if other cell types remain as contaminants in the EBs, an electrophysiological gap between the host CMs can arise after transplantation and trigger fatal arrhythmias such as ventricular tachycardia [36, 37], further stressing the necessity for purity in hPSC-derived CMs. The approach of successive metabolic purification after the MSCS will lower the possibility of arrhythmogenicity after cell transplantation.

**CONCLUSION**

An MSCS is necessary for the effective upscale culture of human CMs and, therefore, the clinical application of hPSC-derived CMs, given the large cell numbers required to treat patients with severe HF. The purification of CMs also remains essential for large-scale culture to minimize the risk of tumor formation. Combining MSCSs and metabolic purification systems will play a pivotal role in achieving routine clinical-grade culture systems for hPSC-derived CMs.

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**AUTHOR CONTRIBUTIONS**

N.H., K.N., and T. Suzuki: collection and assembly of data, data analysis and interpretation, final approval of manuscript; S.T.: collection and assembly of data, data analysis and interpretation, manuscript writing, financial support, final approval of manuscript; H.K.: data analysis and interpretation, final approval of manuscript; F.H., T. Seki, Y.K., A.H., M.O., T.H., S.Y., and M.S.: data analysis and interpretation, final approval of manuscript; R.T.: collection and assembly of data, final approval of manuscript; R.O. and C.F.: administrative support, final approval of manuscript; J.F.: conception and design, data analysis and interpretation, manuscript writing, financial support, final approval of manuscript; K.F.: data analysis and interpretation, financial support, final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicate no potential conflicts of interest.
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