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Supplemental Information

Efficient Large-Scale 2D Culture System for Human Induced Pluripotent Stem Cells and Differentiated Cardiomyocytes

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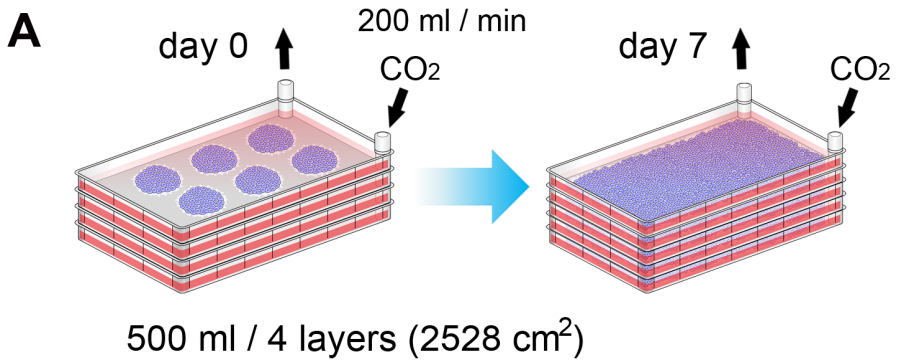
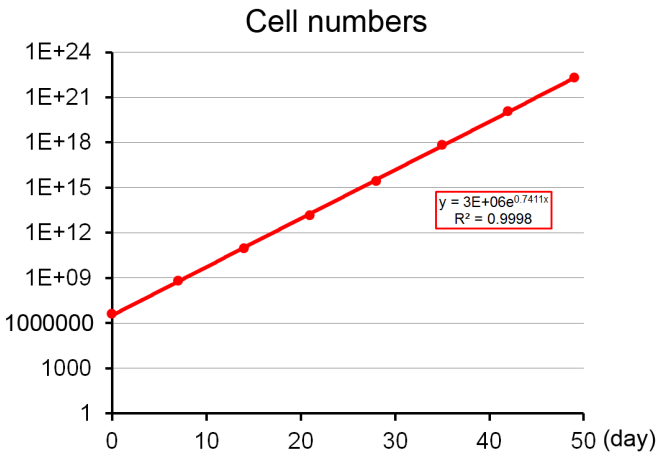
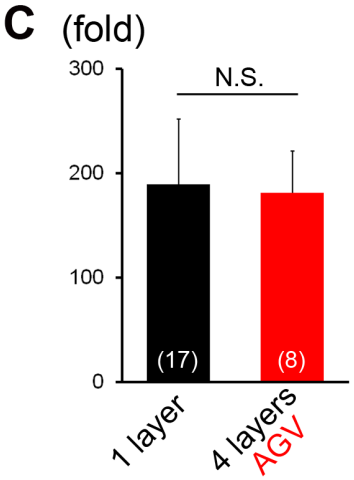
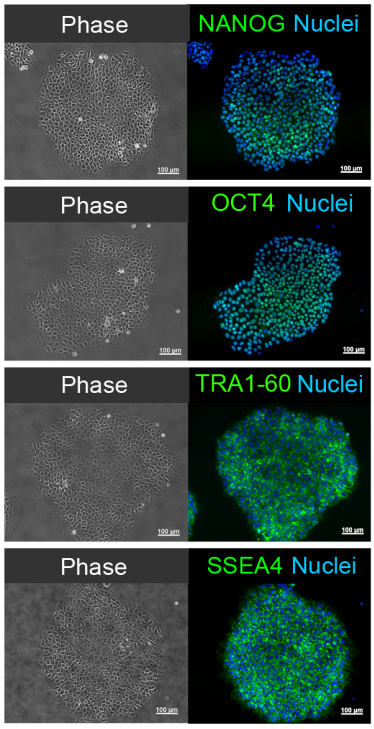
Figure S1


A
day 0
200 ml / min
CO2
day 7
CO2
500 ml / 4 layers (2528 cm2)


B
Cell numbers
1E+24
1E+21
1E+18
1E+15
1E+12
1E+09
1000000
1000
1
y = 3E+06e0.7411x
R² = 0.9998
0
10
20
30
40
50 (day)


C
(fold)
300
200
100
0
N.S.
(17)
(8)
1 layer
4 layers AGV


D
Phase
NANOG Nuclei
Phase
OCT4 Nuclei
Phase
TRA1-60 Nuclei
Phase
SSEA4 Nuclei

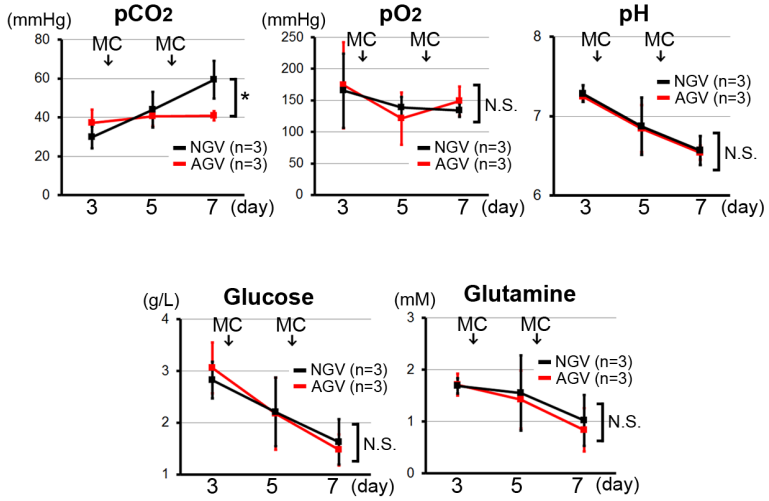

E
(mmHg)
pCO2
MC
MC
NGV (n=3)
AGV (n=3)
*
(day)
(mmHg)
pO2
N.S.
pH
(g/L)
Glucose
(mM)
Glutamine

Figure S2

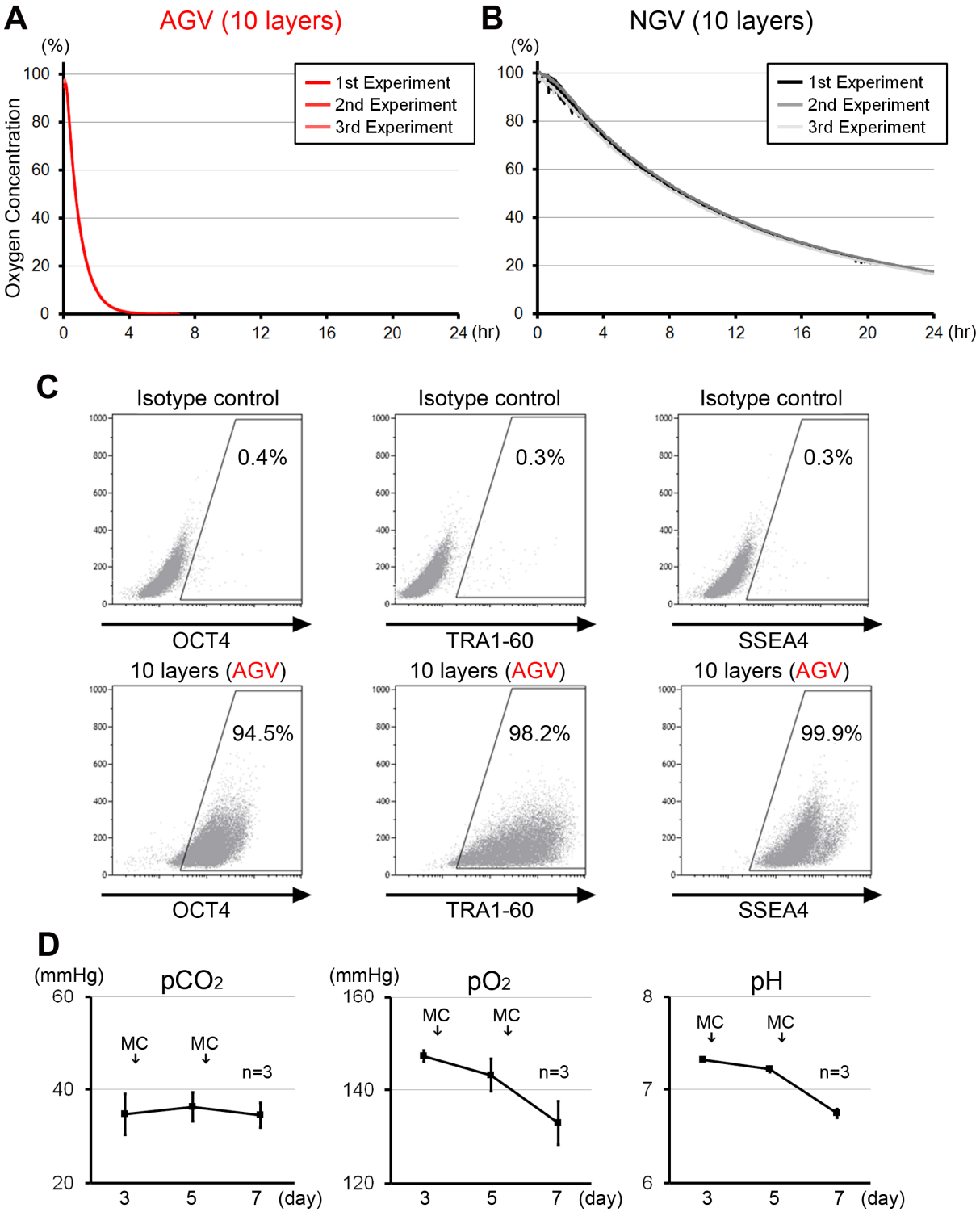
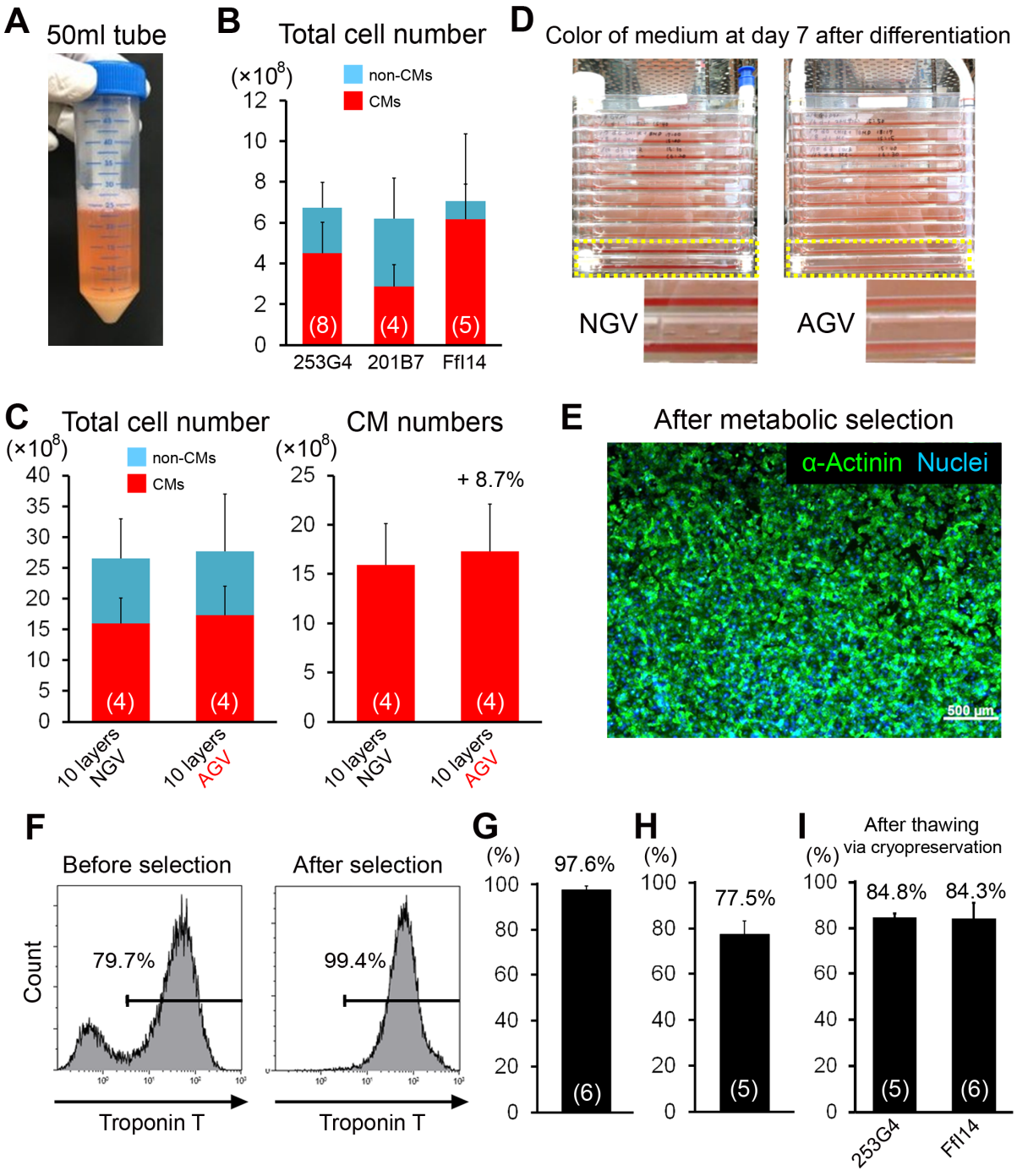

A
AGV (10 layers)
(%)
Oxygen Concentration
1st Experiment
2nd Experiment
3rd Experiment
24 (hr)
B
NGV (10 layers)
C
Isotype control
0.4%
0.3%
OCT4
TRA1-60
SSEA4
10 layers (AGV)
94.5%
98.2%
99.9%
D
pCO2
pO2
pH
(mmHg)
MC
n=3
(day)

Figure S3


A
50ml tube
B
Total cell number
(×10^8)
non-CMs
CMs
12
10
8
6
4
2
0
(8)
(4)
(5)
253G4
201B7
Ffl14
D
Color of medium at day 7 after differentiation
NGV
AGV
C
Total cell number
(×10^8)
non-CMs
CMs
40
35
30
25
20
15
10
5
0
(4)
(4)
10 layers NGV
10 layers AGV
CM numbers
(×10^8)
25
20
15
10
5
0
+ 8.7%
(4)
(4)
10 layers NGV
10 layers AGV
E
After metabolic selection
α-Actinin Nuclei
500 μm
F
Before selection
After selection
Count
79.7%
99.4%
Troponin T
Troponin T
G
(%)
97.6%
100
80
60
40
20
0
(6)
H
(%)
77.5%
100
80
60
40
20
0
(5)
I
After thawing via cryopreservation
(%)
84.8%
84.3%
100
80
60
40
20
0
(5)
(6)
253G4
Ffl14

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. hiPSCs were cultured in a four-layer CP under AGV, related to Figure 1.

(**A**) The scheme of cell culture in a four-layer CP under AGV. The total volume of medium was 500 mL/CP. (**B**) Growth curve of hiPSCs (253G4) in a four-layer CP. Each dot represents a passage of cells. (**C**) The proliferation rate in a four-layer CP (n = 8) under AGV was as much as that in a single layer CP (n = 17). Each bar represents the fold changes from individual passages. (**D**) Immunofluorescent staining showed that hiPSCs expressed the pluripotent markers NANOG, OCT4, TRA1-60, and SSEA4. Bars are 100 μm. (**E**) pCO_2, pO_2, pH, and the concentrations of glucose and glutamine during cultivation in a four-layer CP under NGV or AGV (n = 3 independent experiments). Data are presented as means ± SD. $*p < 0.05$

Figure S2. The AGV system was more efficient for gas exchange than NGV in a 10-layer CP, related to Figure 2.

(**A and B**) Oxygen concentration was measured in the fifth layer of the 10-layer CP under AGV (**A**) and NGV (**B**). (**C**) FACS analyses of pluripotent markers in hiPSCs cultured using 10-layer CPs under AGV. (**D**) pCO_2, pO_2, and pH levels during cultivation in a 10-cm culture dish under NGV (n = 3 independent experiments).

Figure S3. Large-scale culture of cardiac differentiation from hiPSCs in a multilayer CP, related to Figure 3.

(**A**) A pellet of the hiPSC (253G4)-derived cells cultured using one four-layer CP under AGV. (**B**) Total cell number of hiPSC-derived cells in four-layer CPs under AGV (253G4; n = 8, 201B7; n = 4, Ff‌I14; n = 5). Data were obtained from independent experiments. (**C**) Total cell numbers and cardiomyocyte (CM) numbers of hiPSC (Ff‌I14)-derived cells in 10-layer

CPs under NGV or AGV (n = 4 independent experiments). (**D**) Color of medium in hiPSC-derived cells cultured using 10-layer CPs under NGV (left) or AGV (right). (**E**) Representative immunofluorescence staining for α-actinin (green) and nuclei (blue) in hiPSC (FfI14)-derived dispersed cells after metabolic selection. (**F**) Representative FACS analysis for troponin T-positive cells in hiPSC (FfI14)-derived cells before (left) and after (right) metabolic selection. (**G**) The proportion of troponin T-positive CMs in hiPSC (FfI14)-derived dispersed cells after metabolic selection (n = 6 independent experiments). (**H**) Yield-based efficiencies by metabolic selection (n = 5 independent experiments). (**I**) The bar graphs show viability of cryopreserved hiPSC-derived pure cardiomyocytes after thawing (253G4; n = 5, FfI14; n = 6). Data were obtained from independent experiments. Scale bars represent 500 μm (**E**). Data are presented as means ± SD.

Movie S1. Purified hiPSC-derived dispersed CMs, related to Figure 3.

After differentiation using four-layer CPs, hiPSC (253G4)-derived cells were dissociated and seeded on fibronectin-coated single-layer CPs. Then, the cells were metabolically selected under glucose- and glutamine-depleted conditions in the presence of lactate and reseeded.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Maintenance of hiPSCs using single- or multilayer CPs

The hiPSC lines (253G4, 201B7, and FfI14) were obtained from the Center for iPSC Research and Application, Kyoto University. hiPSCs were maintained in modified Stem Fit Medium (Ajinomoto Co., Inc., Japan) on growth factor-reduced Matrigel-coated single- or multilayer (four- or 10-layer) CPs (Thermo Fisher Scientific, USA). In the active gas ventilation (AGV) system, 50 mL/min 5% CO_2 per layer was actively ventilated using a multi-gas incubator (MG-70M; TAITEC Corporation, Japan). hiPSCs were routinely passaged every week using Accutase (Thermo Scientific). Briefly, cells were washed with D-PBS and incubated with Accutase at 37°C. After incubation, cells were collected and reseeded in modified Stem Fit Medium (Ajinomoto) with 10 μM Y27632 (Wako Pure Chemicals, Japan) on growth factor-reduced Matrigel-coated (BD Biosciences, Japan) culture plates. Media were changed every other day (Nakagawa et al., 2014). The number of dissociated single cells was counted using a Vi-Cell cell counter (Beckman Coulter, USA). Karyotypes of hiPSCs were analyzed by Nihon Gene Research Laboratories, Inc. (Sendai, Japan, http://www.ngrljapan.com).

2D cardiomyocyte differentiation using multilayer CPs with NGV or AGV

hiPSCs (1.5×10^7 cells/layer) were seeded in modified Stem Fit Medium (Ajinomoto) on Matrigel-coated multilayer CPs (4 or 10 layers) with NGV or AGV on day -4. When the cells reached 90% confluence on day 0, the medium was changed to RPMI (Wako) with B27 minus insulin (Thermo Scientific), 6 μM CHIR99021 (Wako), and 1 ng/mL bone morphogenic protein 4 (R&D Systems) from day 0 to day 1, as previously reported (Lian et al., 2012; Tohyama et al., 2016), with modifications. The medium was changed to RPMI with B27 minus insulin on day 1. On day 3, the medium was changed to RPMI with B27 minus

insulin and 5 μM IWR1 (Sigma-Aldrich, USA). On day 6, the medium was changed to RPMI with B27 minus insulin. Cells were maintained in MEMα plus 5% fetal bovine serum (FBS; Hyclone) on day 7. From day 7 onwards, the medium was changed every 3–7 days. At day 10 after differentiation, the number of dissociated single cells and cell viability were evaluated by trypan blue staining using a ViCell (Beckman Coulter). Differentiation efficiencies were analyzed using fluorescence-assisted cell sorting (FACS) analysis (Gallios; Beckman Coulter).

Purification of hiPSC-CMs by metabolic selection system

On differentiation days 12–14, hiPSC-derived cells were incubated with D-PBS to remove RPMI with B27 plus insulin for 3 min and then enzymatically dissociated using 0.25% Trypsin-EDTA (Nacalai Tesque). Dissociated cells were collected and seeded on collagen type 1- (IWAKI, Japan) or fibronectin-coated (Sigma) 15 cm dishes or fibronectin-coated single-layer CPs (Thermo Scientific) using MEMα plus 5% FBS. After 1-2 days of cultivation using MEMα plus 5% FBS, hiPSC-derived cells were incubated with D-PBS to remove MEMα plus 5% FBS for 3 min, and the medium was then changed to glucose and glutamine-free DMEM (Ajinomoto) supplemented with 4 mM L-lactic acid and 0.1% BSA (Thermo Scientific) for 3–6 days, as reported previously (Tohyama et al., 2016). The medium was changed every 2 or 3 days to eliminate dead cells. After metabolic selection, purified hiPSC-CMs were cryopreserved or used for immunostaining and FACS analysis.

Components of modified StemFit

In this study, we used modified StemFit medium manufactured by Ajinomoto Co., Inc. As well as StemFit medium (Nakagawa et al., 2014), modified StemFit medium containing 21 amino acids (L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-cyctine, L-

glutamic acid, L-glutamine, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine), 10 vitamins (L-ascorbic acid, cobalamin, biotin, folic acid, I-inositol, niacinamide, D-calcium pantothenate, pyridoxine hydrochloride, riboflavin, and thiamine hydrochloride), five trace minerals (cupric sulfate, ferric sulfate, ferric nitrate, zinc sulfate, and sodium selenite), and growth factors, including basic fibroblast growth factor. Compared with StemFit medium, the quantities of most ingredients were increased for large-scale culture.

Alkaline phosphatase staining

Alkaline phosphatase staining was performed using a Leukocyte Alkaline Phosphatase kit (Sigma) as described previously (Tohyama et al., 2016).

Visualization of gas exchange in a multilayered CP under AGV

A 10-layered culture plate (TAITEC) was filled with 2 L PBS with 20 mg phenol red, and 50 mL/min 5% CO_2 per layer was actively ventilated. A photograph was taken every 10 min.

Measurement of gas exchange rate

In order to measure the gas exchange rate in the AVG system, 500 mL/min N_2 was actively ventilated using the MG-70M multi-gas incubator (TAITEC) through the 10-layered CP. In the NGV system, the 10-layer CP was placed under hypoxic conditions (1% O_2). A sensor tip (SP-PSt3-YAU-D5; PreSens, Germany) and optical fiber (POF-1MSA; PreSens, Germany) were placed in the fifth layer of the 10-layer CP. The oxygen concentration was measured using Fibox 3 (PreSens, Germany).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 20 min. Subsequently, cells were permeabilized with 0.1% Triton X-100 (Sigma) at room temperature for 5 min and then incubated with primary antibodies, i.e., 1:500 dilution of mouse anti-α-actinin (Sigma, A7811), 1:200 dilution of mouse anti-cardiac troponin T (Thermo Scientific, MS-295-p), 1:200 dilution of mouse anti-MLC2a (Synaptic System, Germany, 311011), 1:200 dilution of rabbit anti-MLC2v (ProteinTech, USA, 10906-1-AP), 1:200 dilution of rabbit anti-NANOG (ReproCELL, Japan, RCAB003P), 1:200 dilution of mouse anti-OCT4 (Santa Cruz Biotechnology, USA, sc-5279), 1:200 dilution of anti-TRA1-60 (Millipore, Germany, MAB4360), 1:200 dilution of mouse anti-TRA1-81 (Millipore, MAB4381), and 1:200 dilution of mouse anti-SSEA4 (Millipore, MAB4304), overnight at 4°C. Cells were then washed with PBS containing 0.1% Tween 20 three times prior to incubation with secondary antibodies (Alexa Fluor 488/594 anti-mouse IgG or IgM and Alexa Fluor 488/594 anti-rabbit IgG) for 1 h at room temperature. After nuclear staining with Hoechst 33342 (Thermo Scientific), stained cells were detected by fluorescence microscopy (Axio Observer; Carl Zeiss, Jena, Germany).

FACS analysis

hiPSCs or differentiated CMs were completely dissociated using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) and then fixed with 4% paraformaldehyde for 20 min. Subsequently, cells were permeabilized with 0.1% Triton X-100 (Sigma) at room temperature for 5 min and incubated with primary antibodies, i.e., 1:200 dilution of mouse anti-OCT4 (Santa Cruz Biotechnology, sc-5279), 1:200 dilution of mouse anti-TRA1-60 (Millipore, MAB4360), 1:200 dilution of mouse anti-SSEA4 (Millipore, MAB4304), and 1:200 dilution of mouse anti-cardiac troponin T (Thermo Scientific, MS-295-p), at 4°C overnight. Cells were washed with PBS containing 0.1% Tween 20 prior to incubation with

Alexa 488 donkey anti-mouse IgG secondary antibodies (Thermo Scientific) at room temperature for 2 h. Cells were analyzed by FACS. Negative controls used for gating were based on the cells stained with isotype control antibodies.

Cryopreservation of purified hiPSC-CMs

After purification, the cells were isolated using 0.25% trypsin-EDTA for 10 min at 37°C. The isolated cells were counted using a ViCell (Beckman Coulter) and cryopreserved in STEM-CELLBANKER (Nippon Zenyaku Kogyo Co., Ltd., Japan) using BICELL (Nihon Freezer, Japan). After thawing the hiPSC (253G4 and FfI14)-CMs, cell viabilities were evaluated by trypan blue staining using a Vi-Cell cell counter (Beckman Coulter).

Action potential recordings

After thawing the hiPSC (253G4)-CMs, cells were seeded on fibronectin-coated (Sigma) probes. Then, action potentials were recorded as previously described (Hemmi et al., 2014; López-Redondo et al., 2016). Current-clamp recording was conducted in normal Tyrode's solution containing 135 mM NaCl, 0.33 mM NaH_2PO_4, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.53 mM $MgCl_2$, 5.5 mM glucose, and 5 mM HEPES (pH 7.4 at 35°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 $CaCl_2$ (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 multi-electrode array system

To characterize the functional properties of our purified hiPSC (253G4)-derived CMs, we performed extracellular recording of field potentials (FPs) using a multi-electrode array

(MEA) system (MED 64; AlphaMED Scientific, Japan), as described previously (Egashira et al., 2012; Nakamura et al., 2014; Tanaka et al., 2009). After thawing of hiPSC-derived CMs, cells were seeded on fibronectin-coated (Sigma) probes. The recorded extracellular electrograms were used to determine field potential duration (FPD), defined as the time interval between the initial deflection of the FP and the maximum T wave. FPD measurements were corrected by Bazett's correction formulae (corrected FPD = FPD / [RR interval]$^{1/2}$), where RR indicates the time interval (s) between two consecutive beats. The temperature was maintained at 37°C during these recordings. After incubation for 10–15 min with E4031 (a gift from Esai, Japan) or (-)-isoproterenol hydrochloride (Sigma), the FPs were measured. In experiments using E4031, different channels were analyzed in the MEA probe. In experiments using isoproterenol, different probes were analyzed. The temperature was maintained at 37°C during these recordings.

Statistics

Values are presented as means ± SD or SEM. Statistical significance was evaluated using Student's t tests for comparisons between two mean values. Multiple comparisons between more than three groups were performed using analysis of variance with Dunnett's multiple comparison tests. Results with *p* values of less than 0.05 were considered significant.

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