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Supplemental Information

Purification of human iPSC-derived cells at large scale using microRNA switch and magnetic-activated cell sorting

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Figure S1

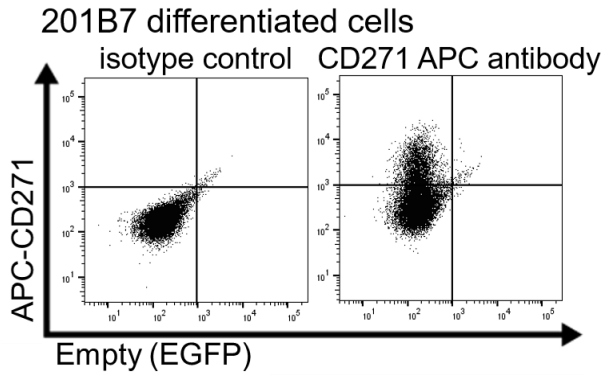

Figure S1. CD271 expression in cells differentiated from the 201B7 iPSC line (related to Figure 2)

Flow cytometric analysis showed that some differentiated cells expressed CD271.

Figure S2

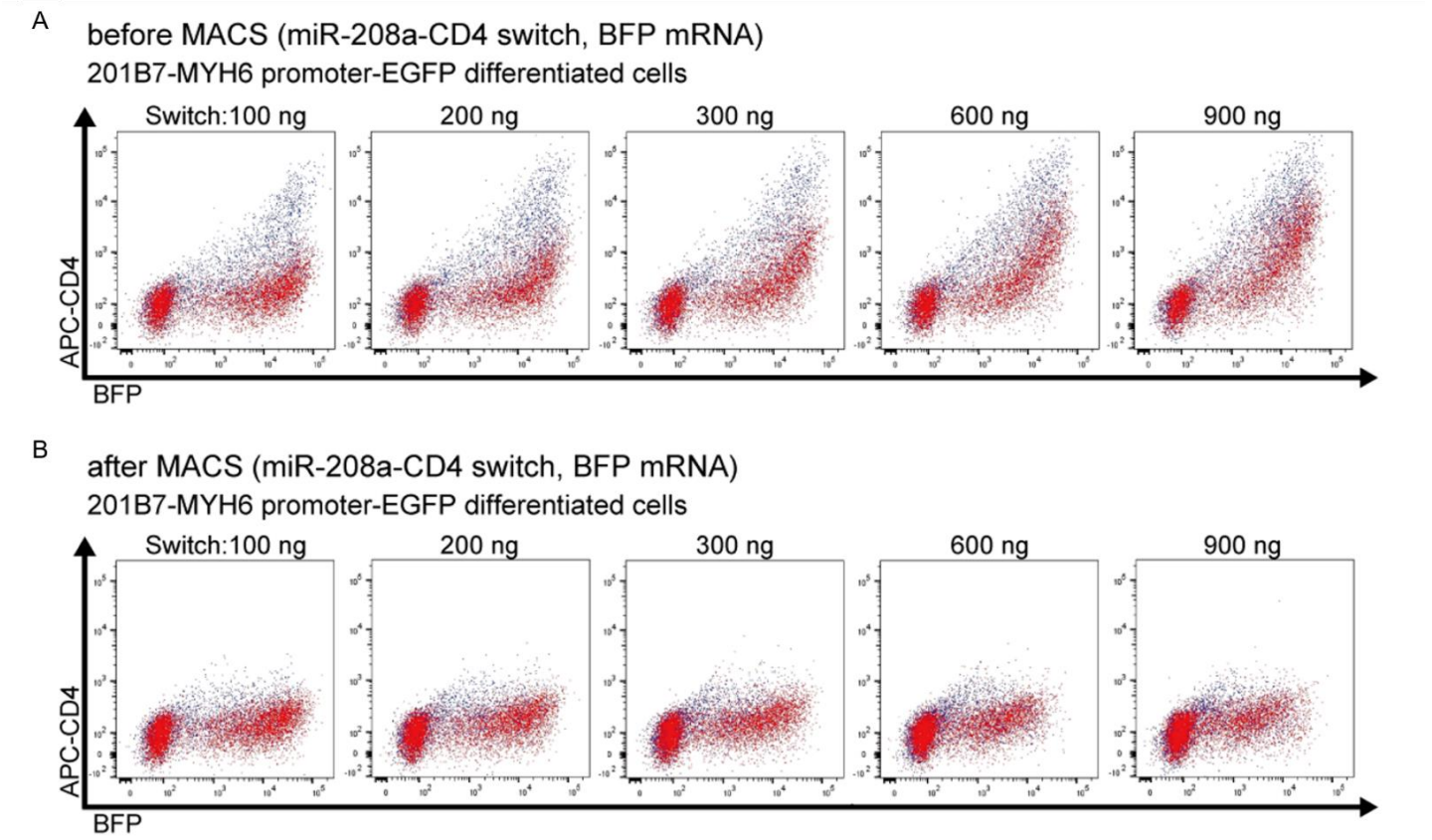

Figure S2. Purification efficiency of CMs depends on amount of miR-208a-CD4 switch (related to Figure 2)

The transfection of 100, 200, 300, 600 and 900 ng of miR-208a-CD4 switch and 300 ng of control BFP mRNA to 201B7-MYH6 promoter-EGFP differentiated cells. Red shows EGFP positive cells (CMs) and blue shows EGFP negative cells (non-CMs). Representative data from two biological independent experiments are shown. (A) Before MACS. As more miRNA switch was transfected, CMs and non-CMs expressed more CD4 on their surface. (B) After MACS. CD4 positive cells were clearly eliminated. Table S2 shows results related to Figure S2.

Figure S3

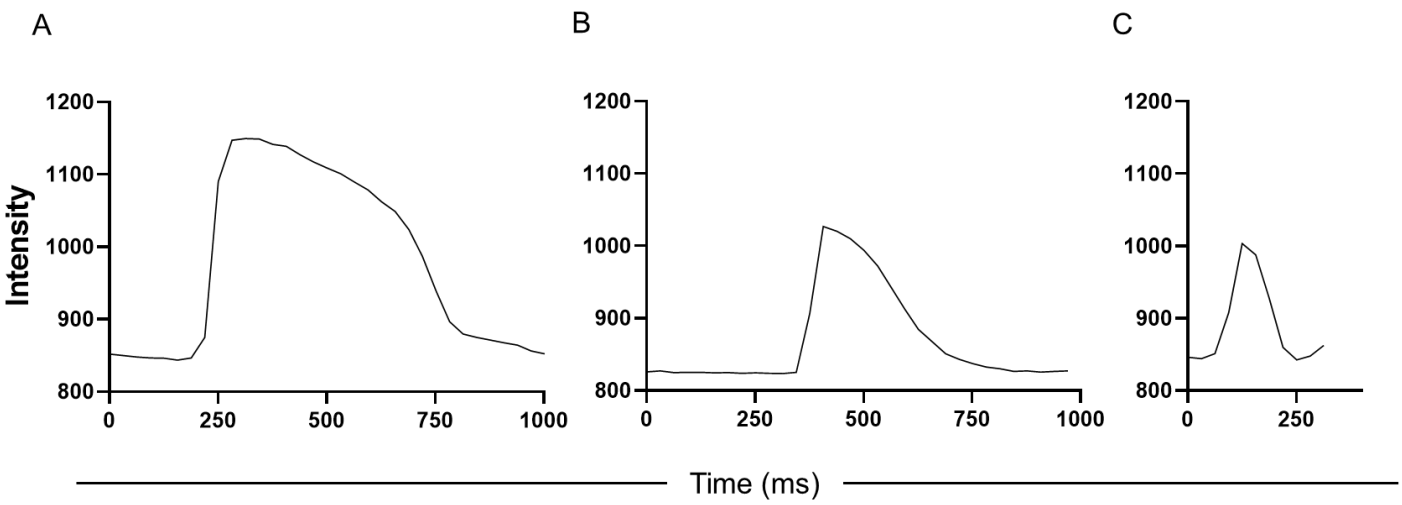

Figure S3. Representative action potentials of the final purified iPSC-CMs. (related to Figures 3 and 6)

The action potential duration (APD) is shown in Table S4. (A) A ventricular action potential with a long plateau phase was observed in the ventricular protocol. (B) An atrial action potential with an absent of a prominent plateau phase was observed in the atrial protocol. (C) A nodal action potential with slow action potential upstroke and phase four depolarization was observed in the nodal protocol.

Figure S4

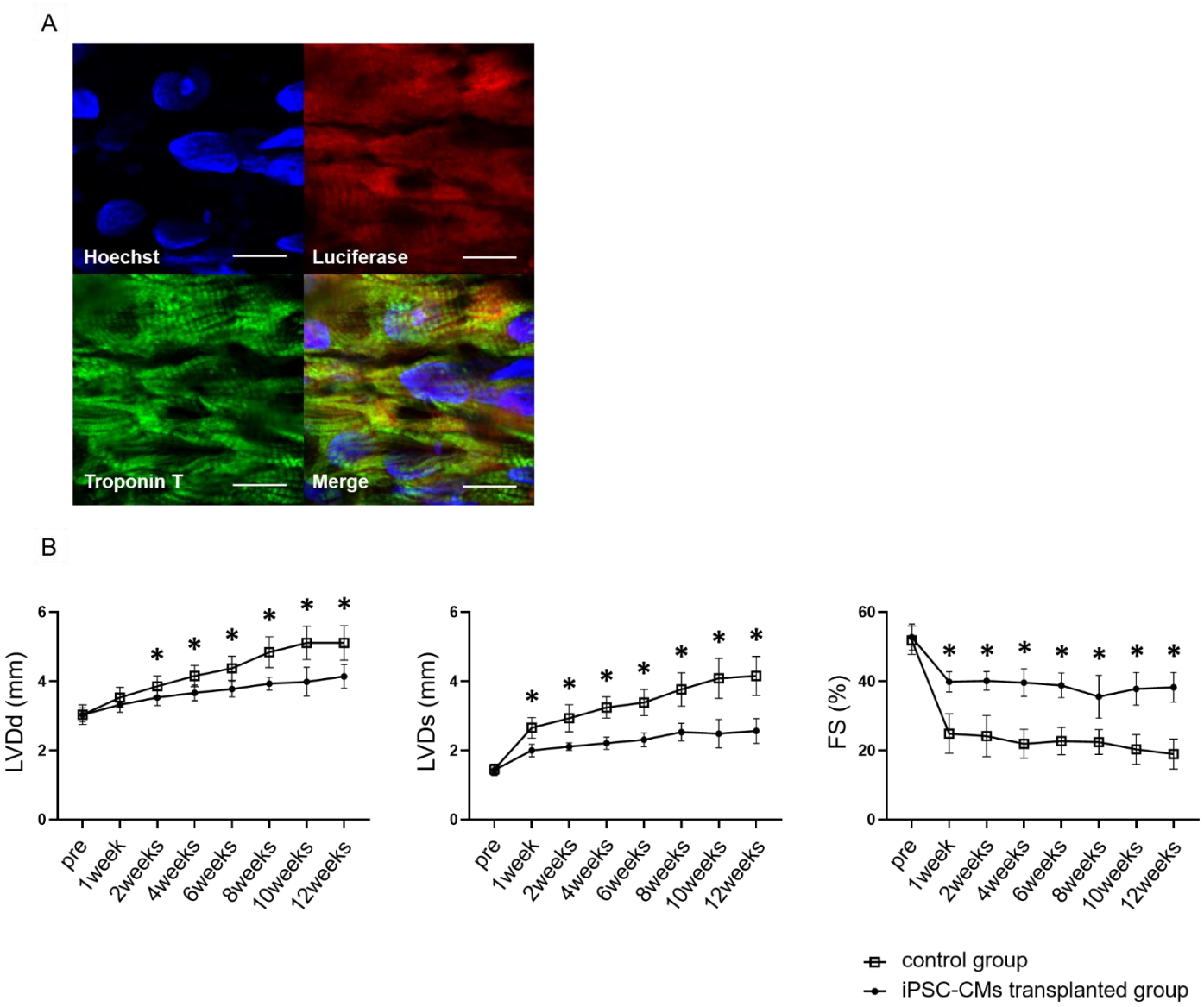

Figure S4. Immunostaining and echocardiographic data of mouse hearts transplanted with purified iPSC-CMs (related to Figure 5)

(A) Immunostaining of Troponin T (green) with high magnification showed that transplanted iPSC-CMs formed sarcomere structures. Scale bars: 10 μm. (B) The cardiac function of the iPSC-CM-transplanted group (n=9) and the control group (n=9) was assessed by echocardiography. LV dimensions (LVDd and LVDs) were increased, and the fractional shortening (FS) was decreased just after the myocardial infarction in both groups. However, compared with the control group, the

degree of changes were relatively small in the iPSC-CM-transplanted group. At one week after the injection, FS was significantly higher in the iPSC-CM-transplanted group compared with the control group. Similarly, LVDd at two weeks after the injection was significantly lower. These differences were maintained throughout the three-months follow-up period. The values are denoted as means. All error bars represent SD. *$P < 0.05$ by the unpaired t test.

Figure S5

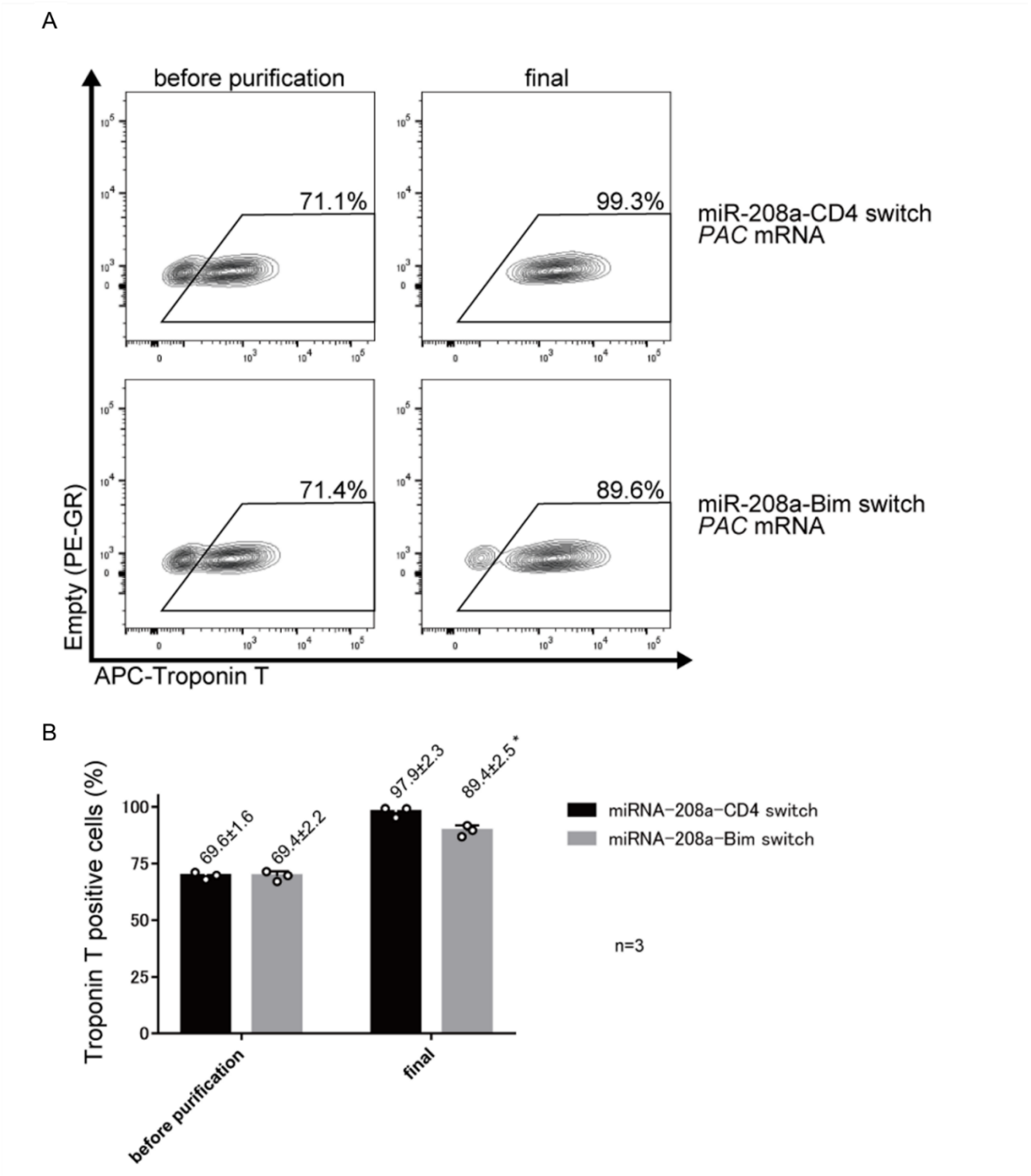

Figure S5. Comparison of miR-208a-CD4 switch and miR-208a-Bim switch (related to Figure 3)

(A) Troponin T positive differentiated 201B7 iPSCs before and after purification (final). Representative data from three biological independent experiments are shown. In the upper panels, 300 ng of miR-208a-CD4 switch and 300 ng of PAC

mRNA were transfected. In the lower panels, 300 ng of miR-208a-Bim switch and 300 ng of PAC mRNA were transfected. Troponin T positive cells are shown in red, and Troponin T negative cells are shown in blue. (B) Troponin T positive differentiated 201B7 iPSCs before purification and after purification. Data are from three biologically independent measurements. The values are denoted as means ± SD. A significant difference was observed in CM purity between the miR-208a-CD4 switch group and miR-208a-Bim switch group (* $P<0.001$, by a two-way ANOVA with repeated measures test). The actual values are shown in Table S6.

Table S1. Time course of mRNA expression (n=3) (related to Figure 2)

Time (days after transfection)		0.5	1	2	3	4	5	6	7	8	9
BFP positive cells (%)	1st	71.2	84.0	77.8	81.0	52.4	43.0	19.9	13.0	11.1	5.9
	2nd	68.4	73.6	81.2	69.6	60.6	32.8	26.6	20.4	17.8	2.8
	3rd	77.2	81.3	84.9	76.5	67.1	38.7	23.5	20.6	18.0	9.4
	mean	72.3	79.6	81.3	75.7	60.0	38.2	23.3	18.0	15.6	6.0
	SD	4.5	5.4	3.6	5.7	7.4	5.1	3.4	4.3	3.9	3.3

Table S2. Purification efficiency of CMs depends on the amount of miRNA switch (n=2) (related to Figure 2 and S2)

	Amount of miRNA switch (ng)	100	200	300	600	900
Before MACS	Transfection efficiency (%)	74.2 ± 1.5	74.3 ± 1.1	76.0 ± 0.4	75.8 ± 2.1	75.5 ± 1.3
	Percentage of CMs (%)	71.4 ± 1.6	71.5 ± 1.1	71.9 ± 1.1	71.2 ± 0.5	71.2 ± 1.8
	Estimated percentage of CMs retained after MACS (%)	68.8 ± 0.9	59.7 ± 1.1	53.2 ± 1.5	38.6 ± 1.5	21.1 ± 2.4
After MACS	The degree of CM purity among successfully transfected cells (%)	89.0 ± 0.2	91.7 ± 0.6	96.6 ± 0.3	97.6 ± 0.2	98.5 ± 0.4

Table S2. Purification efficiency of CMs depends on the amount of miRNA switch (n=2) (related to Figure 2 and S2)

100, 200, 300, 600 and 900 ng of miR-208a-CD4 switch were respectively transfected to 201B7-MYH6 promoter-EGFP differentiated cells with 300 ng of control BFP mRNA. The transfection efficiency is defined as BFP positive cells / all cells. The proportion of CMs is defined as EGFP positive cells / all cells. The estimated proportion of CMs retained after MACS compared with before MACS was calculated as (APC negative + EGFP positive cells) / EGFP positive cells before MACS. After MACS, the degree of CM purity among successfully transfected cells is defined as EGFP positive cells / BFP positive cells. The values are denoted as means ± SD. Data were from two biologically independent measurements.

Table S3. Troponin T positive cells during miR-208a-CD4 switch and MACS purification (n=3) (related to Figure 3)

Troponin T positive cells (%)		before MACS	after MACS	final
201B7	1st	68.8	82.1	98.9
	2nd	75.1	88.8	98.7
	3rd	63.9	82.7	96.1
	mean	69.3	84.5	97.9
	SD	5.6	3.7	1.6
409B2	1st	73.6	83.5	98.2
	2nd	76.5	85.2	98.7
	3rd	67.7	84.2	97.0
	mean	72.6	84.3	98.0
	SD	4.5	0.9	0.9
692D2	1st	70.2	85.1	97.8
	2nd	66.6	82.7	95.2
	3rd	75.6	86.4	98.1
	mean	70.8	84.7	97.0
	SD	4.5	1.9	1.6

Table S4. Electrophysiological characteristics of the final purified CMs (related to Figures 3 and 6)

	APD50 (ms)	APD90 (ms)	APD90 / APD50 (ms)
Ventricular iPSC-CMs	398 ± 6.1	493 ± 38	1.24 ± 0.08
Atrial iPSC-CMs	218 ± 34	385 ± 79	1.76 ± 0.13
Nodal iPSC-CMs	93 ± 1.6	151 ± 2.7	1.62 ± 0.02

Table S4. Electrophysiological characteristics of the final purified CMs (related to Figures 3 and 6)

Action potentials of the final purified iPSC-CMs were recorded using a voltage sensitive fluorescent probe. 10 beats per cells for 10 cells (10 ROIs; a total of 100 beats) were recorded. Action potential durations (APD) at 50% repolarization (APD50) and APD90 were measured, and the ratio of APD90/APD50 was calculated. APD90 / APD50 <1.4, APD90 / APD50 >1.7 and 1.4< APD90 / APD50 <1.7 characterizes ventricular, atrial and nodal action potentials, respectively. Representative recorded action potentials are shown in Figure S3. The values are denoted as means ± SD.

Table S5. Time required to get purified CMs (n=3) (related to Figure 3)

Method	Experiment	Applied cells	Troponin T positive rate before MACS or FACS (%)	Procedural time	Final purified cells	Troponin T positive rate of final purified cells(%)
MACS	1st	3.21×10^8	68.8	28min02sec	1.72×10^8	98.9
	2nd	3.01×10^8	75.1	26min30sec	1.82×10^8	98.7
	3rd	3.42×10^8	63.9	29min58sec	2.00×10^8	97.1
	mean	3.21×10^8	69.2	28min10sec	1.85×10^8	98.2
	SD	0.21×10^8	5.6	104sec	0.14×10^8	0.99
FACS Aria II	1st		68.8	24min56sec	1.12×10^6	98.4
	2nd		75.1	30min32sec	1.20×10^6	99.1
	3rd		63.9	26min08sec	1.14×10^6	97.8
	mean		69.2	27min12sec	1.15×10^6	98.4
	SD		5.6	177sec	0.042×10^6	0.65

Table S5. The required time to get purified CMs (n=3) (related to Figure 3)

In the miRNA-switch-MACS method, $3.21 \pm 0.21\times10^8$ differentiated cells were applied, resulting in $1.85 \pm 0.14\times10^8$ final purified cells after MACS and subsequent puromycin selection. The procedural time was defined as the time for MACS. On the other hand, $1.15 \pm 0.042\times10^6$ purified cells, which is less than 1% that with the miRNA-switch-MACS method, were obtained in FACS method. The procedural time, defined as the time for FACS, is similar to that of MACS. Troponin T positive rates of the final purified cells were similar between methods.

Table S6. Troponin T positive cells using miR-208a-CD4 switch and miR-208a-Bim-switch (n=3) (related to Figure S5)

Troponin T positive cells (%)	miRNA-208a CD4 switch		miRNA-208a Bim switch	
	before purification	final	before purification	final
1st	67.9	95.2	67.1	86.8
2nd	71.1	99.3	71.4	89.6
3rd	69.8	99.2	69.7	91.7
mean	69.6	97.9	69.4	89.4
SD	1.6	2.3	2.2	2.5

Table S7. Chromogranin A and C-peptide positive cells (n=3) (related to Figure 7)

	Chromogranin A positive cells (%)			C-peptide positive cells (%)		
	before MACS	after MACS	final	before MACS	after MACS	final
1st	68.9	87.0	99.7	31.2	43.1	57.7
2nd	73.2	85.4	98.9	29.6	41.2	55.5
3rd	71.4	85.7	99.3	37.3	48.1	65.9
mean	71.2	86.0	99.3	32.7	44.1	59.7
SD	2.2	0.9	0.4	4.1	3.6	5.5

Table S8. Sequences of primers, templates and template DNAs for mRNAs and miR-switches (in reference to STAR methods)

Supplemental experimental procedures

Human iPSC lines, cell culture and pancreatic differentiation

Human iPSC line 585A1 was maintained as previously reported (Hatani et al., 2018). Briefly, iPSCs were maintained by feeder-free cultures with Essential 8 medium (Life Technologies, cat. no. A1517001). iPSCs were induced to differentiate into pancreatic lineage as previously reported with modifications (Kimura et al., 2017; Toyoda et al., 2015). On day 0, iPSCs were dissociated and seeded on Matrigel (Becton Dickinson, cat. no. 354230)-coated plates with RPMI 1640 medium (NACALAI TESQUE, cat. no. 30264-56) in the presence of 2% (vol/vol) B27 supplement (B27, Thermo Fisher Scientific, cat. no. 17504044), 50 U/mL penicillin/streptomycin (P/S, Thermo Fisher Scientific, cat. no. 15140122), 100 ng/mL activin A (R&D Systems, cat. no. 338-AC), 3 μM CHIR99021 (Axon Medchem, cat. no. AXON1386) and 10 μM Y-27632 (Wako, cat. no. 251-00514). After 24 hours, the cells were cultured in RPMI 1640 medium containing 2% B27, 50 U/ml P/S, 100 ng/mL activin A and 1 μM CHIR99021. On day 3, the cells were cultured in RPMI 1640 medium containing 2% B27, 50 U/mL P/S and 100 ng/mL activin A. On day 4, the cells were cultured with Improved MEM Zinc Option (IMEM) medium (Thermo Fisher Scientific, cat. no. 10373017) supplemented with 1% B27, 100 U/mL P/S (IMEM-B27-P/S) and 50 ng/mL keratinocyte growth factor (KGF; R&D Systems, cat. no. 251-KG) for four days. On day 8, the cells were cultured in IMEM-B27-P/S containing 50 ng/mL KGF, 0.5 μM 3-Keto-N-aminoethyl-N'-aminocaproyldihydrocinnamoyl cyclopamine (KAAD-CYC, Toronto Research Chemicals, cat. no. K171000), 10 nM 4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid (TTNPB, Santa Cruz Biotechnology, cat. no. sc-203303) and 100 ng/mL NOGGIN (Pepro-tech, cat. no. 120-10C) for two days. On day 10, the cells were dissociated into single cells and seeded on Matrigel-coated plates with the same medium composition as day 8, except for the addition of 10 μM Y-27632. On day 11, the cells were cultured in IMEM-B27-P/S containing 100 ng/mL KGF, 50 ng/ml epidermal growth factor (EGF, R&D Systems, cat. no. 236-EG), 10 mM Nicotinamide (STEMCELL Technologies, cat. no. 07154) and 50 μM Y-27632 for four days. On day 15, the cells were dissociated into single cells and seeded on low-attachment plates (Greiner, cat. no. 651970) for aggregation cultures with the same medium composition as day 11, except for replacing 50 μM Y-27632 with 10 μM Y-27632. On day 17, the aggregates were cultured in IMEM-B27-P/S containing 1 μM RO4929097 (Selleck, cat. no. S1575), 10 μM Alk 5 inhibitor II (Wako, cat. no. 018-23023), 1 μM Triiodothyronine (Merck Millipore, cat. no. 64245) and 0.1 μM LDN193189 (Wako, cat. no. 124-06011) for four days. From days 21 to 28, the aggregates were cultured with the same medium composition as day 17, except for the removal of RO4929097.

Transplantation of human iPSC-CMs purified by miRNA switch and MACS

All experimental protocols involving animals were approved by the Kyoto University Animal Experimentation Committee, and procedures were performed in accordance with the Guidelines for Animal Experiments of Kyoto University and the Guide for the Care and Use of Laboratory Animals by the Institute of Animal Resources.

A male, 10-week-old NOD/Shi-*scid*/IL-2γ^{null} (NOG) immunodeficiency mouse was intubated and ventilated mechanically under general anesthesia with 2% isoflurane. Myocardial infarction was generated by ligating the left anterior descending artery with 8-0 Prolene (Ethicon, cat. no. EP8730H). 1.0×10^6 purified iPSC-CMs in 20 μL IMDM (Life Technologies, cat. no. 12440-053) were injected directly into the mouse heart by a Hamilton syringe with a 30-gauge needle. We transplanted iPSC-CMs differentiated from 201B7-luc iPSCs, which continuously express luciferase. To confirm the engraftment of iPSC-CMs, bioluminescence imaging was conducted. Mice were anesthetized with inhaled isoflurane, and D-luciferin (SPI, cat.no. XLF-1) was administered at a dose of 200 mg/kg i.p.

Images of the mice were captured using an in vivo bioluminescence imaging system (IVIS, Caliper Life Sciences). In the control mice, 20 μL IMDM alone was injected.

Before surgery and every two weeks thereafter, the mice were mildly anesthetized with inhaled isoflurane, and their left-ventricular dimensions (LVDd and LVDs) were measured using transthoracic echocardiography (GE Vivid S, GE). The fractional shortening (FS) was calculated as 100×(LVDd-LVDs) / LVDd (%) as an index of cardiac function.

Immunostaining and flow cytometry

Immunostaining was performed using the following primary antibodies: rabbit anti-cardiac Troponin T (abcam, cat. no. ab45932; 1:400), mouse anti-cardiac Troponin T (Thermo Scientific, cat. no. MS-295-P; 1:200), goat anti-luciferase (Promega, cat. no. G7451; 1:100), rabbit anti-MLC2V (Proteintech, cat. no. 10906-1-AP; 1:500), mouse anti-COUTPF-Ⅱ (R&D Systems, cat. no. PP-H7147-00; 1:200), rabbit anti-NKX2.5 (Cell Signaling Technology, cat. no. 8792; 1:800), rabbit anti-Chromogranin A (Abcam, cat. no. ab68271, 1:500), rat anti-C-peptide (DSHB, cat. no. GN-ID4, 1:200), mouse anti-glucagon (Sigma-Aldrich, cat. no. G2654; 1:200), rabbit anti-somatostatin (Dako, cat. no. A0566: 1:200), and goat anti-PDX1 (R&D Systems, cat. no. AF2419; 1:200). The secondary antibodies used in this study were as follows: goat anti-mouse IgG-Alexa Fluor 546 (Life Technologies, cat. no. A-11030; 1:400), goat anti-rabbit IgG-Alexa Fluor 488 (Life Technologies, cat. no. A-11034; 1:400), donkey anti-rabbit IgG-Alexa Fluor 488 (Life Technologies, cat. no. A-21206; 1:400), donkey anti-mouse IgG-Alexa Fluor 546 (Life Technologies, cat. no. A-10036; 1:400), donkey anti-rat IgG-Alexa Fluor 647 (Jackson, cat. no. 712-605-150; 1:400), donkey anti-goat IgG-Alexa Fluor 488 (Life Technologies, cat. no. A-11055; 1:400). Hoechst 33342 (Life Technologies, cat. no. H3570; 1:10000) was used to counterstain nuclei. The stained cells were visualized using a confocal microscope (FV1000, Olympus).

For the preparation of flow cytometer samples, we used the following antibodies: APC mouse anti-human CD4 (BD Biosciences, cat. no. 340443; 1:100), APC mouse anti-human CD31 (Biolegend, cat. no. 303116; 1:30), Alexa Fluor 647 mouse anti-human CD49a (Biolegend, cat. no. 328310; 1:20), APC mouse anti-human CD90 (BD Pharmingen, cat. no. 559869; 1:50), APC mouse anti-human CD140b (Biolegend cat. no. 323608; 1:10) PE/Cy7 mouse anti-human CD172a/b (Biolegend, cat. no. 323808; 1:20), mouse anti-cardiac Troponin T (Thermo Scientific, cat. no. MS-295-P; 1:200), and APC goat anti-mouse IgG (BD Biosciences, cat. no. 550826; 1:100). APC mouse IgG1 k isotype control (BD pharmingen, cat. no. 554681; 1:50) and PE/Cy7 mouse IgG1 k isotype control (BD pharmingen, cat. no. 557872; 1:50) were used as controls.

To measure the proportion of CMs, staining was carried out on cells fixed with 4% paraformaldehyde in PBS and done in PBS with 2% FCS and 0.5% saponin (Sigma, cat. no. 84510). Stained cells were analyzed and sorted using FACSAria II (BD Biosciences). Data were analyzed using FlowJo v10.6.1 analysis software (BD Biosciences).

Recording action potentials

To record action potentials, 2×10^5 iPSC-CMs were placed on a 35-m glass bottom dish, and the medium was exchanged with Gey's Balanced Salt Solution (Sigma-Aldrich, cat. no. G9779) and 0.1% Fluo Volt (Thermo Fisher Scientific, cat. no. F10488), which is a voltage-sensitive fluorescent probe. After 20 minutes of incubation at 37℃, the medium was exchanged with Gey's Balanced Salt Solution without Fluo Volt, and the sample was incubated for another hour at 37℃. Thereafter, the action potentials were recorded using AquaCosmos software (Hamamatsu Photonics). The recordings were performed under electrical stimulation at 1 Hz by Master-9 (AMPI) in the ventricular and atrial protocols, and under spontaneous beating condition in the nodal protocol.

Quantitative PCR

Total RNA was extracted using RNeasy Mini Kit (QIAGEN) after lysing the cells with QIAzol Lysis Reagent (QIAGEN). Extracted RNA was reverse transcribed into cDNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO). A qPCR analysis was performed with TaqMan probes (Thermo Fisher Scientific). The samples were analyzed using StepOnePlus Real-Time PCR System (Applied Biosystems). The fold change of the gene expressions was calculated using the ddCt method. The TaqMan probes were GAPDH (Assay ID; Hs99999905_m1), MYL2 (Assay ID; Hs00166405_m1), IRX4 (Assay ID; Hs00212560_m1), MYL7 (Assay ID; Hs01085598_g1), NR2F2 (COUPTF-Ⅱ) (Assay ID; Hs00819630_m1), SHOX2 (Assay ID; Hs01061127_m1), and ISL1 (Assay ID;Hs00158126_m1).

Supplemental references

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