SUPPLEMENTAL FIGURES AND LEGENDS

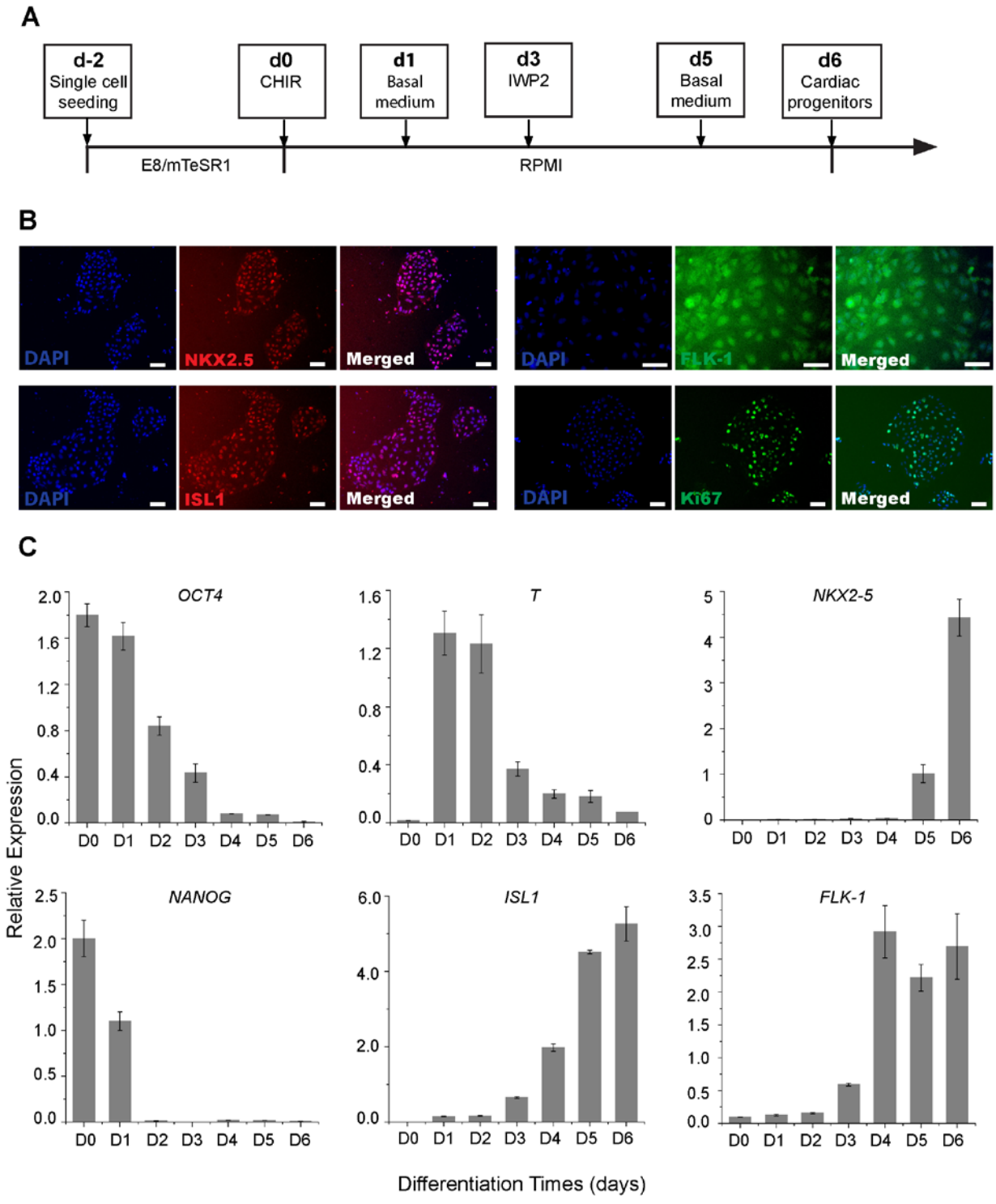

Figure S1. Albumin-free differentiation of NKX2.5+ISL1+FLK-1+ cardiac progenitors from hPSCs by small molecule modulation of Wnt signaling. (A) Schematic of the protocol for defined, albumin-free differentiation of hPSCs to cardiac progenitors in RPMI medium. (B) H13 hESCs were differentiated as illustrated in (A), re-passaged on day 6 and immunostained with indicated markers on day 7. Scale bars, 100 μm. (C) H13 hESCs were differentiated as illustrated in (A) and developmental gene expression was assessed by quantitative RT-PCR at indicated time points. Data are represented as mean ± SEM of three independent replicates.

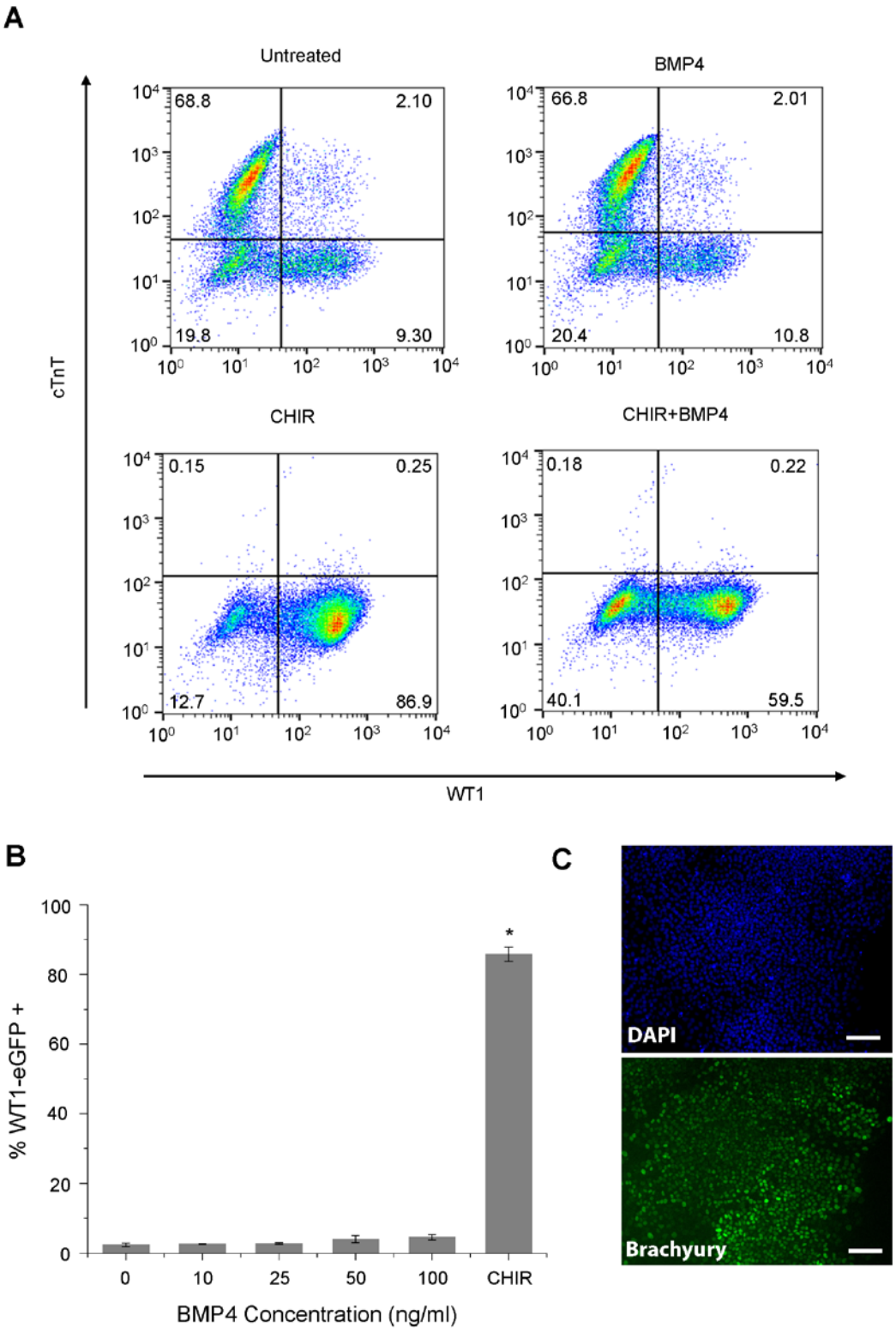
A
Untreated
BMP4
CHIR
CHIR+BMP4
68.8
2.10
19.8
9.30
66.8
2.01
20.4
10.8
0.15
0.25
12.7
86.9
0.18
0.22
40.1
59.5
cTnT
WT1
B
% WT1-eGFP +
BMP4 Concentration (ng/ml)
0
10
25
50
100
CHIR
*
C
DAPI
Brachyury

Figure S2. Flow cytometry analysis of cTnT and WT1 expression in day 12 hPSC-derived cell cultures. (A) Representative flow plot of H13 hESC-derived day 12 cultures differentiated as shown in Fig. 1A in the presence or absence of 2 μM CHIR99021 and 10 ng/mL BMP4. (B) eGFP expression in day 12 ES03 WT1-2A-eGFP hESC-derived cultures differentiated as shown in Fig. 1A with the indicated BMP4 concentration; 2 μM CHIR is a positive control. * $p<0.05$, CHIR treatment and indicated BMP4 concentration versus 0 ng/ml BMP4. Data are represented as mean ± SEM of three independent replicates. (C) H9 hPSCs were treated with 5 ng/ml BMP4 for 2 days and representative immunostaining images of brachyury are shown. Scale bars, 100 μm.

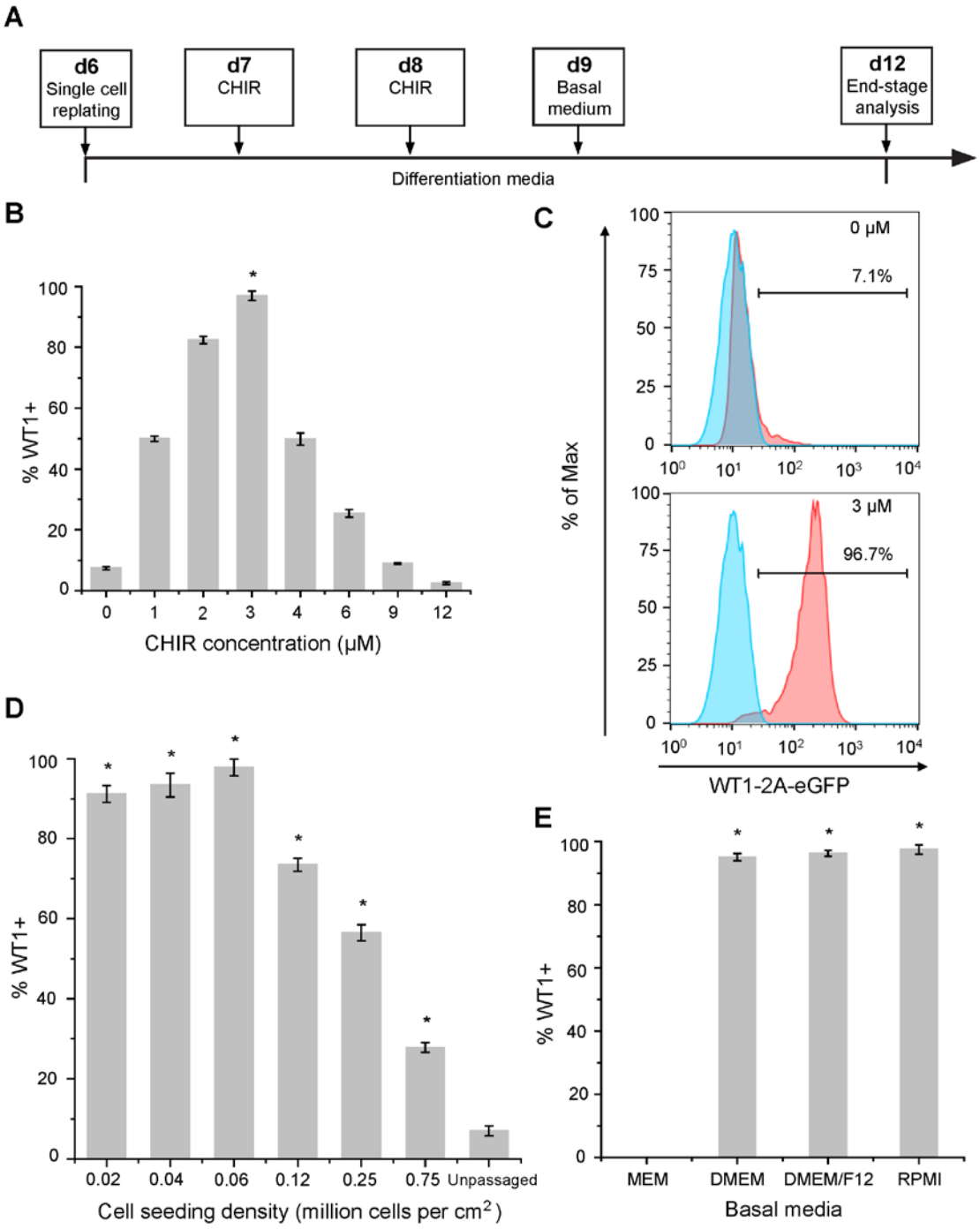
A
d6
Single cell replating
d7
CHIR
d8
CHIR
d9
Basal medium
d12
End-stage analysis
Differentiation media
B
% WT1+
CHIR concentration (μM)
C
0 μM
7.1%
3 μM
96.7%
% of Max
WT1-2A-eGFP
D
% WT1+
Cell seeding density (million cells per cm²)
Unpassaged
E
% WT1+
MEM
DMEM
DMEM/F12
RPMI
Basal media

Figure S3. Chemically-defined, albumin-free conditions to generate WT1+ epicardial cells. (A) Schematic of the protocol for chemically-defined differentiation of hPSC-derived cardiac progenitors to WT1+ epicardial cells via GSK3 inhibition. (B) H13 hESC-derived day 6 cardiac progenitor cells were cultured as illustrated in (A) in LaSR basal medium with indicated CHIR99021 (CHIR) concentrations. At day 12, cells were analyzed for WT1 expression by flow cytometry. * $p<0.05$, 3 μM CHIR99021 versus all other indicated CHIR concentrations. (C) Representative flow analysis of WT1-eGFP knockin ES03-derived epicardial cells differentiated with the indicated CHIR concentration. (D) H13 hESC-derived day 6 cardiac progenitor cells were cultured as illustrated in (A) with the indicated day 6 cell seeding density in LaSR basal medium. At day 12, cells were analyzed for WT1 expression by flow cytometry. * $p<0.05$, indicated cell seeding density versus unpassaged control. (E) H13 hESC-derived day 6 cardiac progenitor cells were seeded at a density of 0.06 million cells/cm^2 and cultured as illustrated in (A) with 3 μM CHIR in the indicated basal media. At day 12, cells were analyzed for WT1 expression by flow cytometry. * $p<0.05$, indicated medium versus MEM. Data are represented as mean ± SEM of five independent replicates.

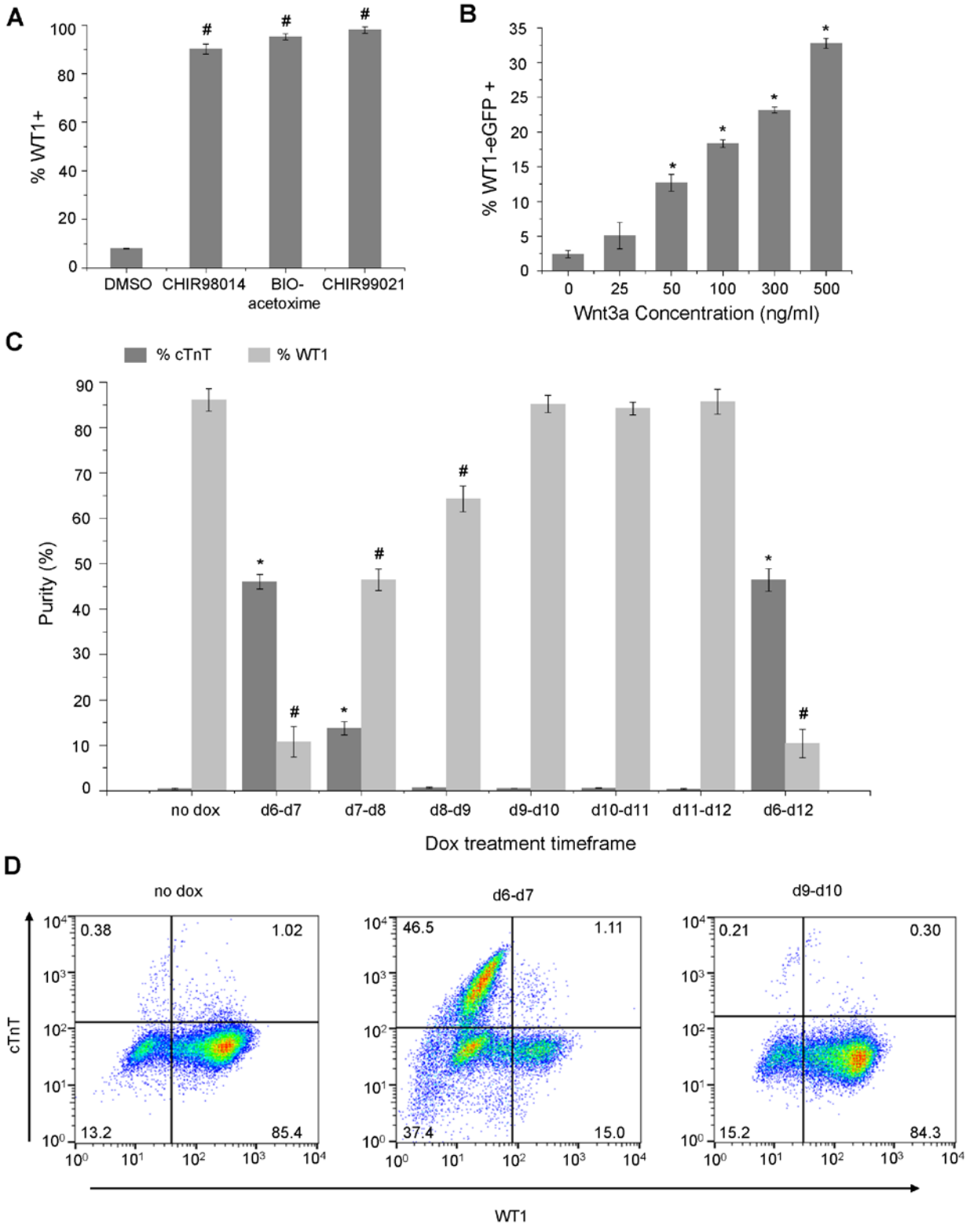
A
% WT1+
DMSO
CHIR98014
BIO-acetoxime
CHIR99021
B
% WT1-eGFP +
Wnt3a Concentration (ng/ml)
C
% cTnT
% WT1
Purity (%)
no dox
d6-d7
d7-d8
d8-d9
d9-d10
d10-d11
d11-d12
d6-d12
Dox treatment timeframe
D
no dox
d6-d7
d9-d10
cTnT
WT1
0.38
1.02
13.2
85.4
46.5
1.11
37.4
15.0
0.21
0.30
15.2
84.3

Figure S4. Epicardial cell differentiation from hPSC-derived cardiac progenitors is β-catenin dependent. (A) Day 6 H13 hESC-derived cardiac progenitor cells, generated as shown in Fig. S1A, were seeded at a density of 0.06 million cells/cm^2, differentiated as illustrated in Fig. S3A in LaSR basal medium with DMSO, 0.3 μM CHIR98014, 0.3 μM BIO-acetoxime or 3 μM CHIR99021 from day 7 to day 9, and subjected to flow cytometry analysis of WT1 expression at day 12. # $p<0.05$, indicated treatment versus DMSO. (B) Day 6 ES03 WT1-2A-eGFP hPSC-derived cardiac progenitor cells were seeded at a density of 0.06 million cells/cm^2, differentiated as illustrated in Fig. S3A in LaSR basal medium with the indicated Wnt3a treatment from day 7 to day 9, and subjected to flow cytometry analysis of eGFP expression at day 12. * $p<0.05$, indicated Wnt3a concentration versus 0 ng/ml. (C-D) 19-9-11 ishcat-1 iPSC-derived day 6 cardiac progenitor cells were differentiated as illustrated in Fig. S3A with 2 μg/ml doxycycline addition at the indicated times. At day 12, cells were analyzed for WT1+ and cTnT+ expression by flow cytometry (C). Representative flow cytometry analysis of cTnT and WT1 expression with the indicated dox treatments are shown in (D). Data are represented as mean ± SEM of five independent replicates. * $p<0.05$, cTnT expression with indicated dox treatment versus no dox treatment. # $p<0.05$, WT1 expression with indicated dox treatment versus no dox treatment.

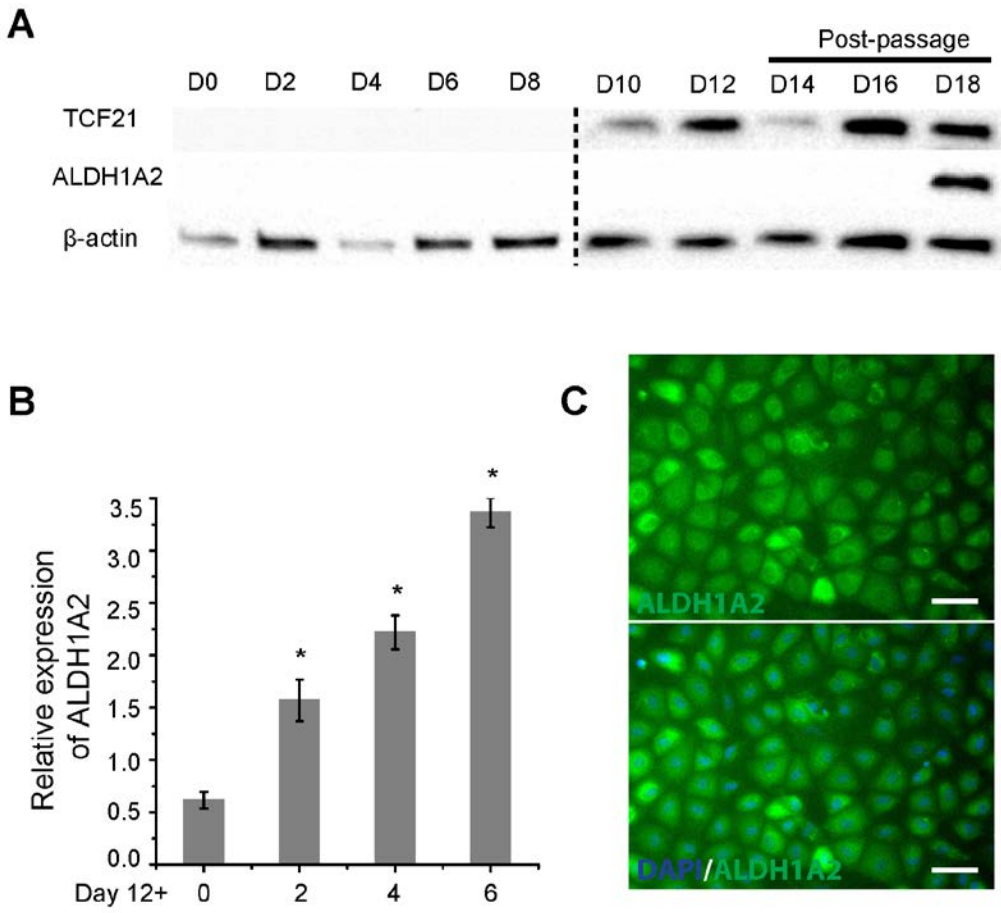

Figure S5. Epicardial cells mature after passage at a low density in chemically-defined medium. H13 hESC-derived day 6 cardiac progenitor cells were seeded at a density of 0.06 million cells per cm^2 as illustrated in Fig. 3A in RPMI/Vc/Ins medium with 3 μM CHIR99021 from day 7 to day 9. At different time points, TCF21 and ALDH1A2 (A) expression was assessed by western blot. After passage at a density of 0.05 million cells per cm^2 on day 12, differentiated cultures were subjected to qPCR (B) and immunostaining (C) analysis of ALDH1A2. Scale bars, 50 μm. Data are represented as mean ± SEM of three replicates. * $p<0.05$, ALDH1A2 expression at indicated time points vs. day 12.

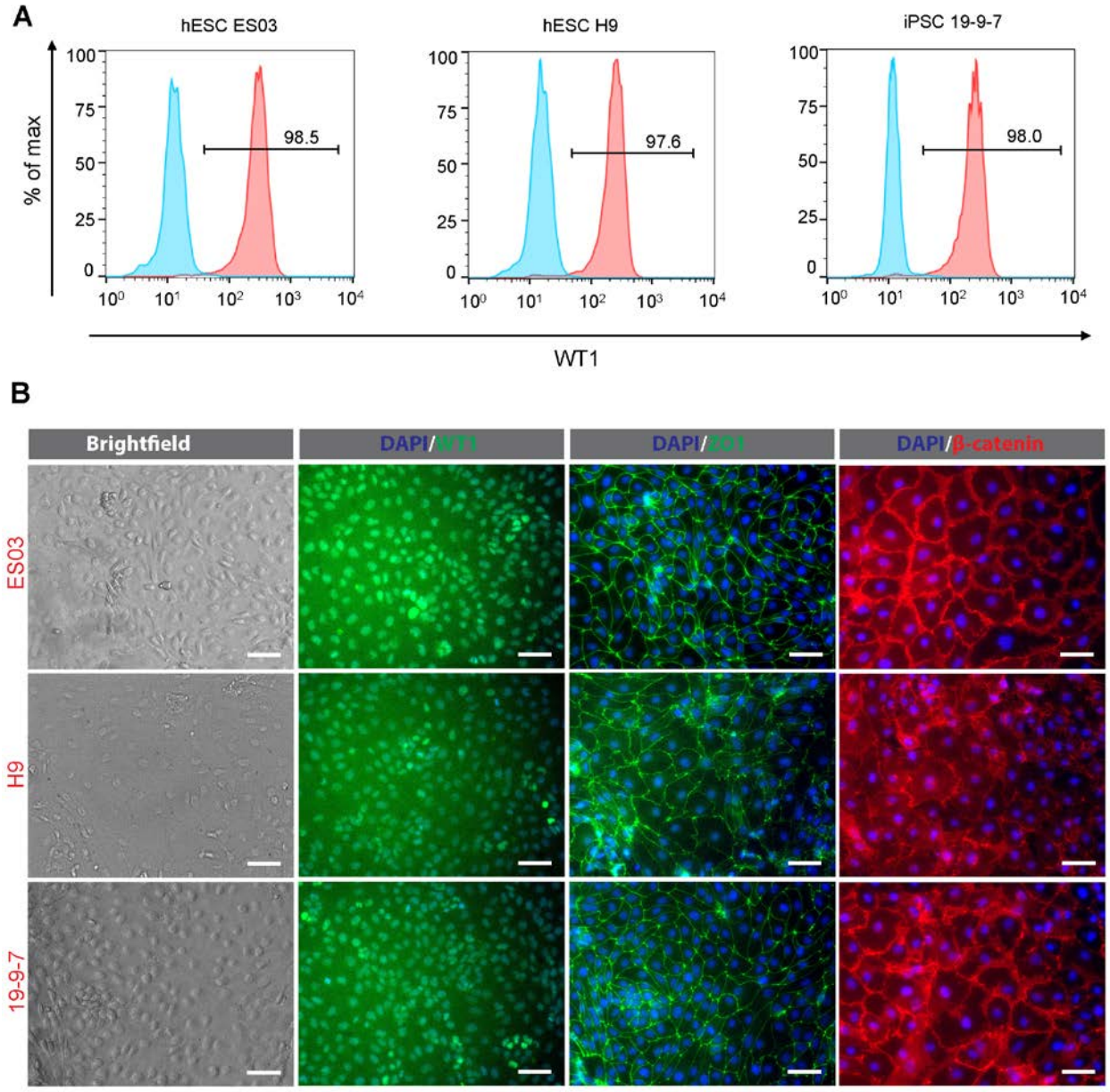

Figure S6. Differentiation of multiple hESC and iPSC lines to epicardial cells. Epicardial cells were generated as described in Fig. 3A from different hPSC lines: hESC H9, hESC ES03, iPSC 19-9-7. Day 12 pro-epicardial cells were subjected to flow cytometry analysis of WT1 expression (A), and representative contrast and immunostaining images of WT1, ZO1 and β-catenin of post-passage day 18 epicardial cells are shown in (B). Scale bars, 50 μm.

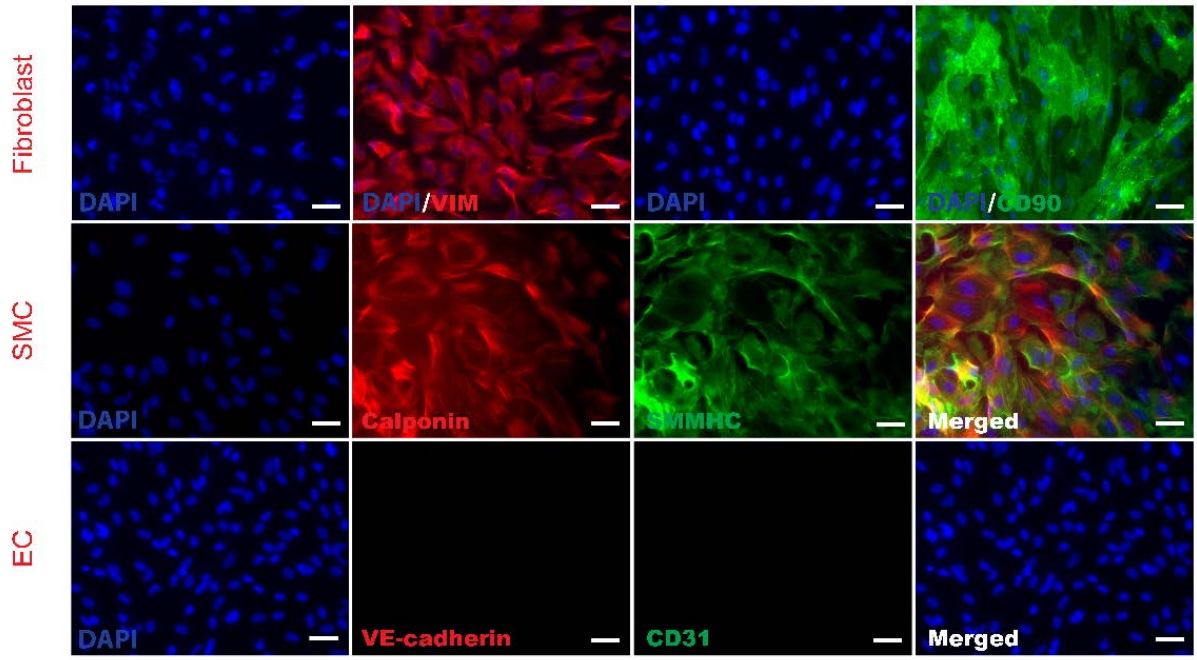

Figure S7. hPSC-derived epicardial cells underwent EMT in response to bFGF and TGF-β1 treatment. Immunostaining analysis of indicated fibroblast, smooth muscle and endothelial cell markers in LaSR basal medium with bFGF and bFGF+TGF-β1, and EGM-2 medium, respectively. EC: Endothelial cells; SMC: smooth muscle cells. Scale bars, 50 μm.

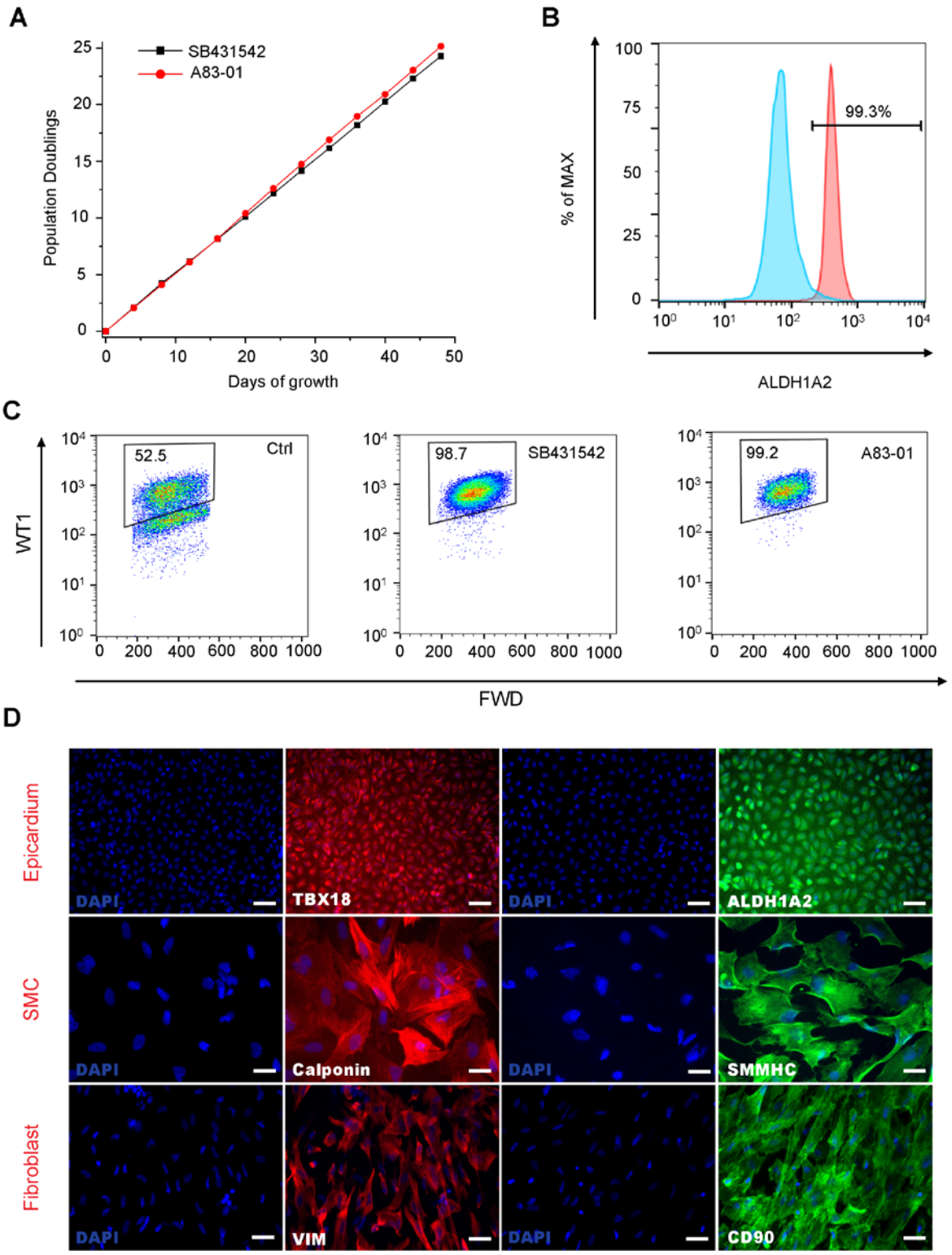
A
SB431542
A83-01
Population Doublings
Days of growth
B
% of MAX
99.3%
ALDH1A2
C
WT1
52.5
Ctrl
98.7
SB431542
99.2
A83-01
FWD
D
Epicardium
SMC
Fibroblast
DAPI
TBX18
ALDH1A2
Calponin
SMMHC
VIM
CD90

Figure S8. Long-term maintenance of 19-9-11 iPSC-derived epicardial cells. hPSCs were differentiated to epicardial cells as illustrated in Fig. 3A, passaged and counted every four days in the presence of the indicated TGF-β inhibitors: 0.5 μM A83-01 or 2 μM SB431542. The population doublings were calculated and shown in (A), and day 48 cultures were subjected to flow analysis of ALDH1A2 (B) and WT1 (C) expression. (D) Immunostaining analysis showed day 48 expanded epicardial cells retained strong expression of TBX18 and ALDH1A2, and the potential to differentiate into SMMHC+Calponin+ smooth muscle cells and VIM+CD90+ fibroblasts. Scale bars, 50 μm.

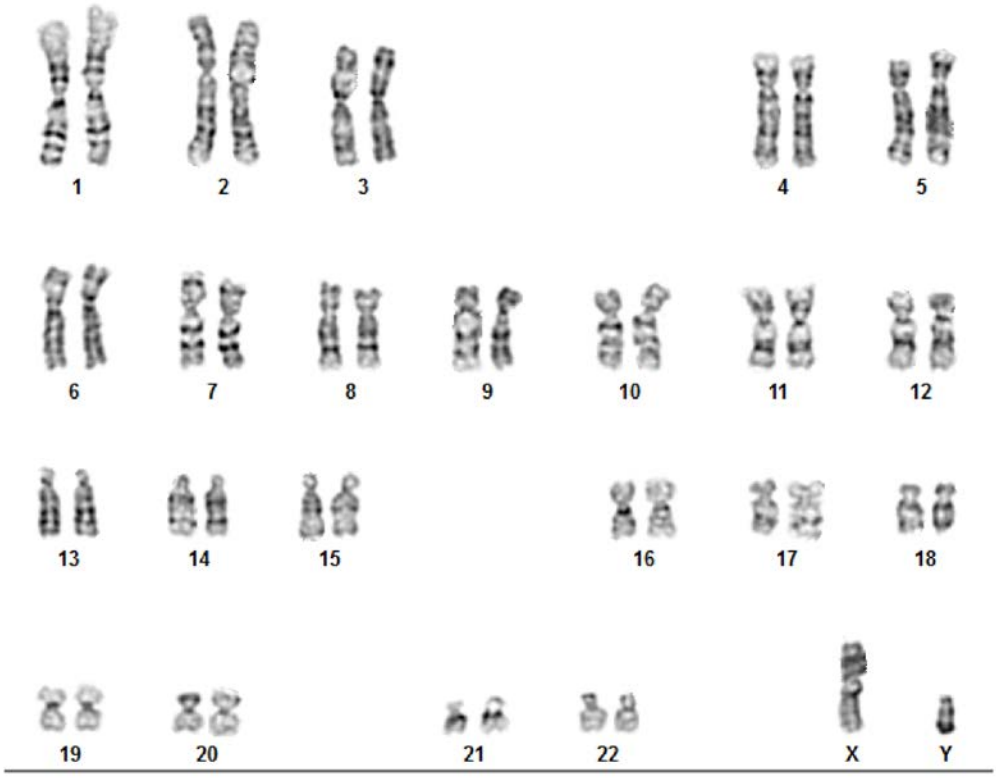

Figure S9. Chromosome stability of hPSC-derived epicardial cells after long-term culture. Representative karotype analysis of H13 hESC-derived day 50 epicardial cells cultured in A83-01 containing medium. 5/5 cells karyogrammed retained a normal karyotype.

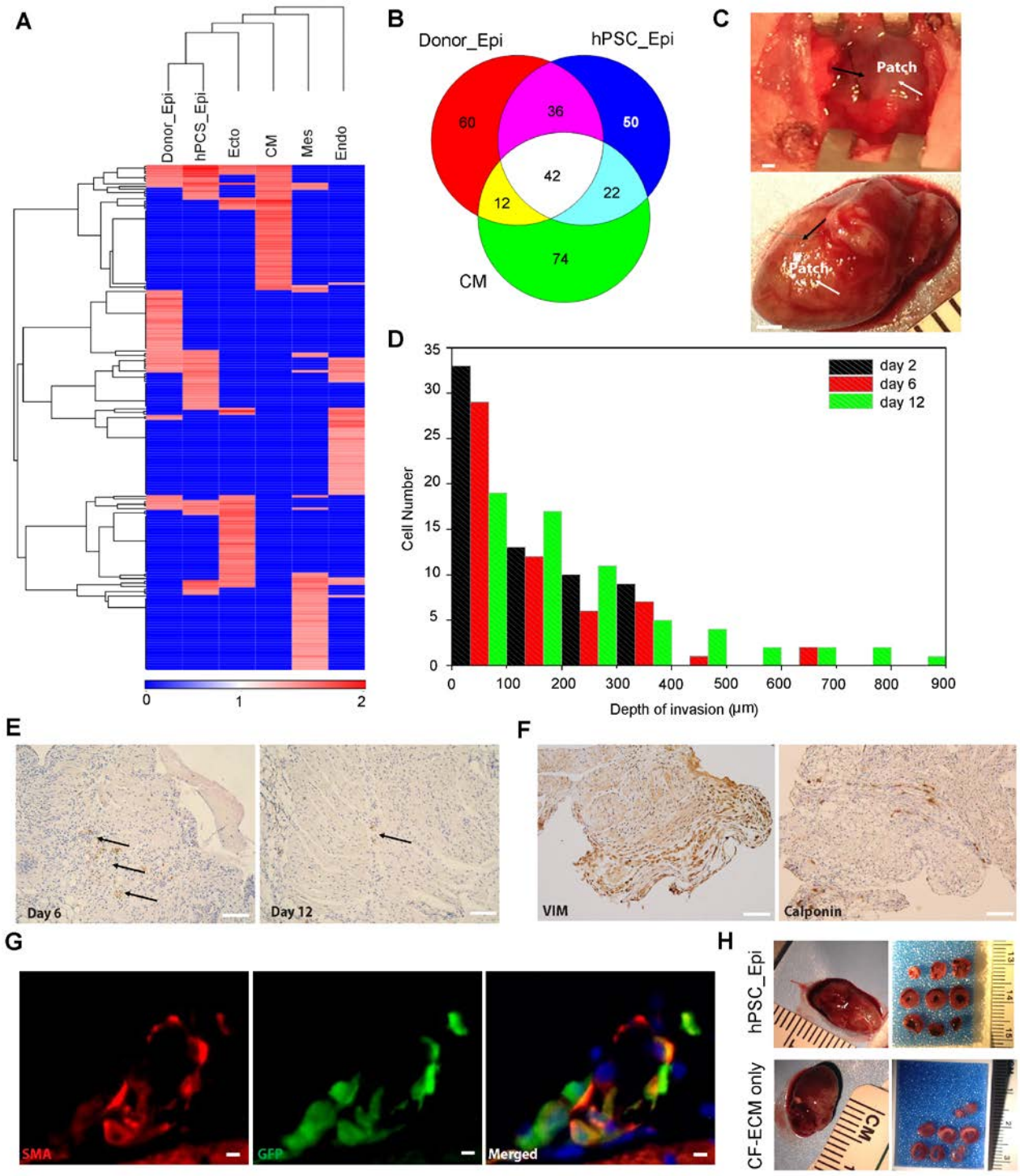
A
Donor_Epi
hPCS_Epi
Ecto
CM
Mes
Endo
0
1
2
B
Donor_Epi
hPSC_Epi
CM
60
36
50
42
12
22
74
C
Patch
Patch
D
day 2
day 6
day 12
Cell Number
Depth of invasion (μm)
E
Day 6
Day 12
F
VIM
Calponin
G
SMA
GFP
Merged
H
hPSC_Epi
CF-ECM only

Figure S10. hPSC-derived epicardial cells were functionally similar to primary epicardial cells both *in vitro* and *in vivo*. (A) Hierarchical clustering of the top 50 significantly enriched pathways in indicated cell types. The color bar indicates the absolute normalized enrichment score (NES) for the enriched pathways. (B) Venn diagram showing the number of pathways which were enriched in different cell types (relative to hPSCs). The top 150 significantly enriched pathways ($p<0.05$), ranked by absolute NES for each cell type were used for analysis. (C) The cardiac fibroblast-derived extracellular matrix (CF-ECM) patch seeded with cells was photographed at time of placement (top) and 12 days after transplantation (bottom). Black arrows denote the sutures and white arrows denote the CF-ECM scaffold. Scale bars, 1 cm. Histogram distribution of depth of invasion (D) and representative photomicrographs (E) of eGFP-positive cells in 6-, and 12-day heart tissue sections are shown. Arrows denote the eGFP positive cells. (F) After 12 days, hearts were harvested and representative vimentin (VIM) and calponin stains of cross-sections of the heart are shown. Scale bars, 50 μm. (G) Representative immunostaining images of smooth muscle actin (SMA) and GFP are shown. Scale bars, 200 μm. (H) Non-tumorigenic potential of hPSC-derived epicardial cells after long-term culture. ES03-eGFP hESC-derived epicardial cells seeded onto CF-ECM patch were transplanted to the mouse heart and harvested for dissection after 12 days. A CF-ECM patch lacking epicardial cells was performed as a control.

SUPPLEMENTAL TABLES

Table S1. Signaling modulators used in this study

Modulator	Targeted pathway	Concentration
bFGF	FGF	10 ng/mL
BMP4	BMP	0~100 ng/mL
Dorsomorphin (DM)	BMP	4 μM
Wnt3a	Wnt	0~500 ng/mL
IWP2	Wnt	5 μM
CHIR99021 (CHIR)	Wnt	1~9 μM
CHIR98014	Wnt	0.3 μM
BIO-acetoxime	Wnt	0.3 μM
Purmorphamine (PURM)	Hedgehog	2 μM
Retinoic acid (RA)	RA	2 μM
PD0325901 (PD)	MEK	0.5 μM
Verteporfin (VP)	Hippo pathway	1 μM
RO4929097 (RO)	Notch	2 μM
TGF-β1	TGF-β	5 ng/mL
TGF-β1 antibody (α-TGF-β I)	TGF-β	200 μg/mL
TGF-β pan antibody (TGF-β Pan)	TGF-β	200 μg/mL
A83-01	TGF-β	0.5 μM
RepSox	TGF-β	0.5 μM
SB505124	TGF-β	2 μM
SB431542	TGF-β	2 μM

Table S2. Purity and yield of WT1+ epicardial cells at day 12

Cell line	Purity of WT1+ cells (%)	Yield of WT1+ cells (10^5/cm^2)
ES03	95.74 ± 1.38	3.79 ± 0.09
ES03-WT1-eGFP	94.74 ± 0.99	3.59 ± 0.24
H9	93.72 ± 1.63	3.67 ± 0.04
H13	96.88 ± 1.01	3.70 ± 0.19
19-9-7	96.90 ± 0.86	3.82 ± 0.11
19-9-11	97.82 ± 0.88	3.87 ± 0.05

Data are presented as mean ± SD of three independent experiments.

Table S3. Top 15 Gene Annotations enriched in donor epicardial cells compared to hPSCs

GO Description	NES	p-value
Positive regulation of I-kappaB kinase NF kappaB cascade	2.068	0.000
Regulation of I-kappaB NF kappaB cascade	1.983	0.000
Endoplasmic reticulum part	1.900	0.000
Endoplasmic reticulum membrane	1.810	0.000
I-kappaB kinase NF kappaB cascade	1.804	0.000
Intrinsic to organelle membrane	1.803	0.000
Positive regulation of signal transduction	1.800	0.000
ER to golgi vesicle mediated transport	1.787	0.009
Extracellular matrix part	1.782	0.000
Keratinocyte differentiation	1.768	0.020
Hematopoietin interferon class D200 domain cytokine receptor binding	1.765	0.000
Integral to endoplasmic reticulum membrane	1.765	0.016
ER golgi intermediate compartment	1.763	0.005
Integral to organelle membrane	1.759	0.000
Intrinsic to endoplasmic reticulum membrane	1.758	0.005

* NES: normalized enrichment score. P-value of 0 means <0.0001.

Table S4. Top 15 Gene Annotations enriched in hPSC-derived epicardial cells compared to hPSCs

GO Description	NES	p-value
Sulfuric ester hydrolase activity	1.776	0.004
Extracellular matrix	1.760	0.000
Proteinaceous extracellular matrix	1.751	0.000
Extracellular matrix part	1.727	0.002
Muscle development	1.709	0.000
Negative regulation of cell cycle	1.695	0.002
Collagen	1.668	0.0052
Cell cycle arrest GO 0007050	1.662	0.0032
Muscle cell differentiation	1.649	0.011
Skeletal development	1.637	0.000
Skeletal muscle development	1.604	0.008
Keratinocyte differentiation	1.600	0.009
Myoblast differentiation	1.594	0.010
Striated muscle development	1.576	0.010
Contractile fiber part	1.572	0.021

* NES: normalized enrichment score. P-value of 0 means <0.0001.

Table S5. Top 15 Gene Annotations enriched in hPSC-derived cardiomyocytes (CMs) compared to hPSCs

GO Description	NES	p-value
Regulation of heart contraction	2.070	0.000
Structural constituent of muscle	1.971	0.000
Muscle development	1.890	0.000
Mitochondrial membrane part	1.842	0.000
Myosin complex	1.832	0.000
Growth factor activity	1.819	0.002
Mitochondrial membrane	1.817	0.000
Energy derivation by oxidation of organic compounds	1.803	0.000
Heart development	1.781	0.000
Mitochondrial inner membrane	1.774	0.000
Mitochondrial respiratory chain	1.758	0.000
Mitochondrial envelope	1.739	0.000
Contractile fiber part	1.696	0.002
Generation of precursor metabolites and energy	1.665	0.000
Extracellular matrix	1.664	0.000

* NES: normalized enrichment score. P-value of 0 means <0.0001.

Table S6. Antibodies used in this study

Antibody	Source/Isotype/clone /cat. no.	Concentration
Smooth muscle actin	Lab Vision/Mouse IgG2a/ 1A4 /ms-133-p	1:100 (IS)
Cardiac troponin T	Lab Vision/Mouse IgG1/ 13-11 /ms-295-p1	1:200 (FC& IS)
ISL1	DSHB/Mouse IgG2b/39.4D5-s	1:20 (IS)
NKX2.5	Santa Cruz/Rabbit IgG/sc-14033/H-114	1:100 (IS)
Flk-1	Santa Cruz/Mouse IgG1/sc-6251/A-3	1:200 (IS)
Ki67	BD Biosciences/Mouse IgG1/550609	1:100 (IS)
WT1	Abcam/Rabbit IgG/ab89901	1:250 (FC & IS)
TCF21	Sigma-Aldrich/Rabbit IgG/HPA013189	1:200 (IS)
TBX18	Sigma-Aldrich/Rabbit IgG/HPA029014	1:200 (IS)
ALDH1A2	Sigma-Aldrich/Rabbit IgG/HPA010022	1:50 (FC & IS)
Brachyury	R&D Systems/Goat IgG/AF2085	1:200 (IS)
ZO1	Invitrogen/Rabbit IgG/402200	1:200 (IS)
β-catenin	Cell Signaling/Mouse IgG1/2698/L87A12	1:200 (IS)
Vimentin	Sigma-Aldrich/Mouse IgG1/V6630/V9	1:200 (IS)
VE-cadherin	Santa Cruz/Mouse IgG1/F-8/sc9989	1:100 (IS)
CD31	ThermoFisher/Rabbit IgG/RB-10333-P	1:100 (IS)
CD90	BD Pharmingen/Mouse IgG1/559869	1:200 (IS)
E-cadherin	BD Biosciences/Mouse IgG2a/560061	1:200 (IS)
SMMHC	Abcam/Rabbit IgG/ab82541	1:800 (IS)
Calponin	Abcam/Mouse IgG1/ab700/CALP	1:200 (IS)
GFP	DSHB/Mouse IgG1/12E6	1:20 (FC & IS)
GFP	Abcam/Rabbit IgG/ab6556	1:1000 (IS)
Mitochondria (human specific)	Millipore/Mouse IgG1/113-1/MAB1273	1:100 (IS)
β-actin	Cell Signaling/Rabbit mAb(HRP Conjugate)/5152S/13E5	1:5,000(WB)
Secondary Antibody	Alexa 488 Chicken anti-Gt IgG/A-21467	1:1,000
Secondary Antibody	Alexa 488 Chicken anti-Rb IgG/A-21441	1:1,000
Secondary Antibody	Alexa 488 Goat anti-Ms IgG1/A-21121	1:1,000
Secondary Antibody	Alexa 488 Goat anti-Rb IgG/A-11008	1:1,000
Secondary Antibody	Alexa 594 Goat anti-Ms IgG2b/A-21145	1:1,000
Secondary Antibody	Alexa 594 Goat anti-Rb IgG/A-11012	1:1,000
Secondary Antibody	Alexa 647 Goat anti-Ms IgG2b/A-21242	1:1,000
Secondary Antibody	Alexa 647 Goat anti-Rb IgG/A-21244	1:1,000

Table S7. Oligonucleotide primers used in this study

Genes	Sequences (5' - 3')	Size (bp)/Tm (°C)
OCT4	**F:** CAGTGCCCGAAACCCACAC	161/58
	R: GGAGACCCAGCAGCCTCAAA	
NANOG	**F:** CGAAGAATAGCAATGGTGTGACG	328/58
	R: TTCCAAAGCAGCCTCCAAGTC	
T	**F:** AAGAAGGAAATGCAGCCTCA	101/58
	R: TACTGCAGGTGTGAGCAAGG	
ISL1	**F:**CACAAGCGTCTCGGGATT	202/58
	R:AGTGGCAAGTCTTCCGACA	
FLK-1	**F:** GTGACCAACATGGAGTCGTG	218/60
	R: TGCTTCACAGAAGACCATGC	
NKX2.5	**F:**GCGATTATGCAGCGTGCAATGAGT	220/58
	R:AACATAAATACGGGTGGGTGCGTG	
TNNT2	**F:** TTCACCAAAGATCTGCTCCTCGCT	165/58
	R:TTATTACTGGTGTGGAGTGGGTGTGG	
TBX18	**F:**CCCAGGACTCCCTCCTATGT	200/59
	R:TAGGAACCCTGATGGGTCTG	
WT1	**F:**CAGCTTGAATGCATGACCTG	200/60
	R:GATGCCGACCGTACAAGAGT	
TCF21	**F:**ACCCTCTTCCTCGCTTTCTC	180/59
	R:TGCTCTCGTTGGAAGTCACA	
ALDH1A2	**F:**CTCCTCTGTCACACCCCATT	198/59
	R:TTGACAGCTGGAAAGATGGA	
SNAI2	**F:** ACAGAGCATTTGCAGACAGG	147/59
	R: GTGCTACACAGCAGCCAGAT	
CDH1	**F:** TTCTGCTGCTCTTGCTGTTT	142/59
	R: TGGCTCAAGTCAAAGTCCTG	
CDH2	**F:** CTCCAATCAACTTGCCAGAA	136/58
	R: ATACCAGTTGGAGGCTGGTC	
CTNNB1	**F:** GAATGAGACTGCTGATCTTGGAC	250/58
	R: CTGATTGCTGTCACCTGGAG	
GAPDH	**F:** GTGGACCTGACCTGCCGTCT	152/58
	R: GGAGGAGTGGGTGTCGCTGT	
WT1 KI (Red)	**F:** GGTCTTGGTTTCTGCTGGAC	2777/60
	R: AAGTCGTGCTGCTTCATGTG	
WT1 KI (Blue)	**F:** TGAAAAGCCCTTCAGCTGTC	204 or 2847/60
	R: TGAGGAGGAGTGGAGAGTCAG	

LEGENDS FOR SUPPLEMENTAL MOVIES

Movie S1, H13 hESC-derived day 6 cardiac progenitors were differentiated with 3 μM CHIR99021 from day 7 to day 9 as described in Fig. 1A in LaSR basal medium. Movie S1 shows day 12 non-contracting pro-epicardial cells.

Movie S2, H13 hESC-derived day 6 cardiac progenitors were differentiated with 10 ng/mL BMP4 from day 7 to day 9 as described in Fig. 1A in LaSR basal medium. Movie S2 shows day 12 spontaneously contracting cardiomyocytes.

Movie S3, 19-9-11 ishcat-1 iPSC-derived day 6 cardiac progenitors were differentiated with 2 μg/ml doxycycline addition from day 6 to day 7 as described in Fig. 3A in RPMI/Vc/Ins medium. Movie S3 shows day 12 spontaneously contracting cardiomyocytes.

Movie S4, 19-9-11 ishcat-1 iPSC-derived day 6 cardiac progenitors were differentiated with no doxycycline treatment as described in Fig. 3A in RPMI/Vc/Ins medium. Movie S4 shows day 12 non-contracting pro-epicardial cells.