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Supplemental Information

Expandable Cardiovascular Progenitor Cells Reprogrammed from Fibroblasts

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Inventory of Supplemental Information

1. Supplemental Figures and Legends

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SUPPLEMENTAL FIGURES AND LEGENDS

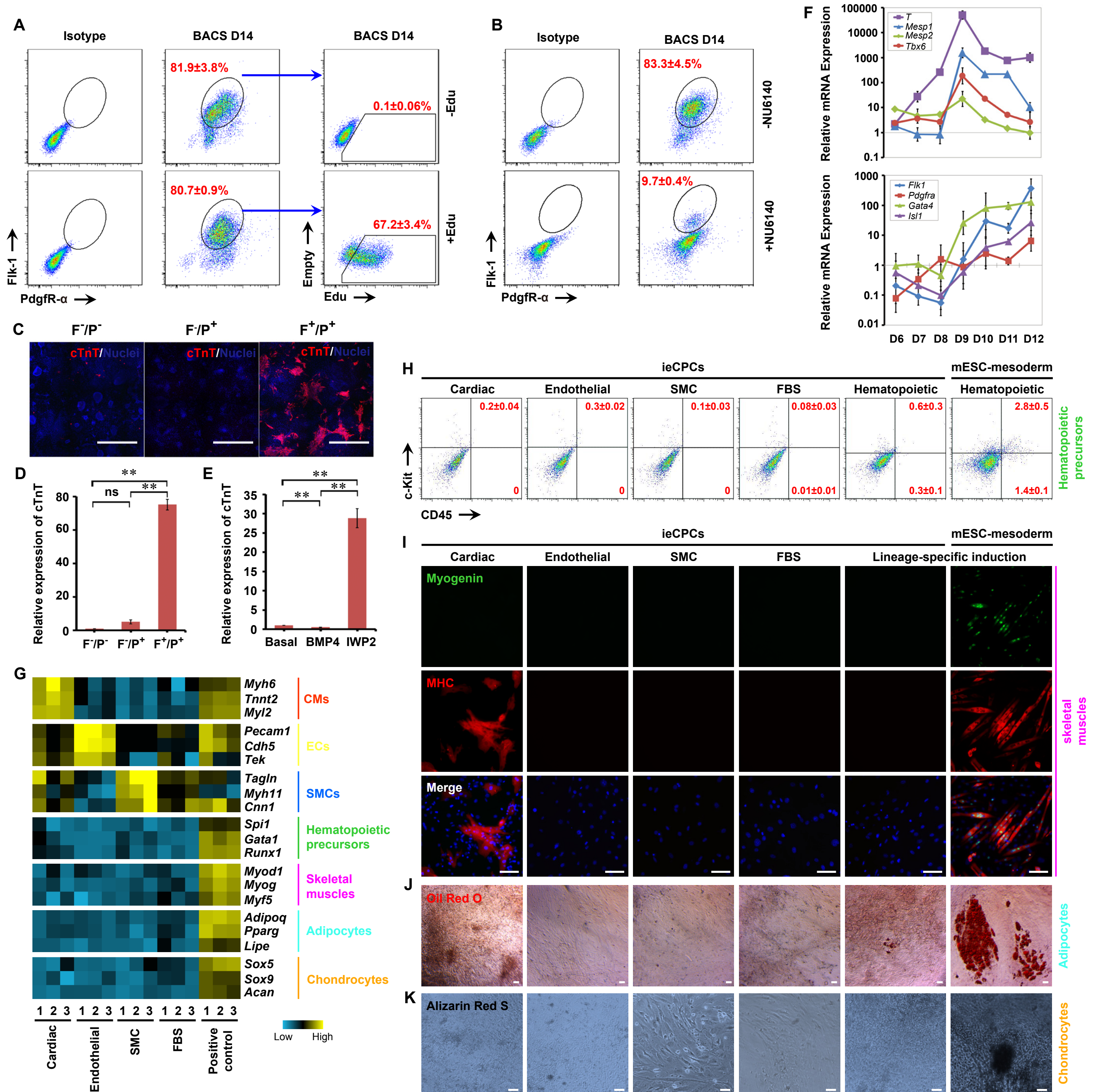


Figure S1. Further Characterization of ieCPC Generation, Related to Figure 1.

(A) Flow-cytometry analysis of the percentage of Edu⁺ cells in Flk-1⁺/PdgfR-α⁺ (F⁺/P⁺) cells on day 14.

(B) Flow-cytometry analysis of the percentage of F⁺/P⁺ cells on day 14 in cells with or without 4 μM NU6140 treatment from days 8–14.

(C) Immunofluorescence analyses of cardiomyocyte (CM) marker cTnT in cells differentiated from Flk-1⁻/PdgfR-α⁻ (F⁻/P⁻), Flk-1⁻/PdgfR-α⁺ (F⁻/P⁺), and F⁺/P⁺ cells in the precense of 5 μM IWP2. Scale bars, 1 mm.

(D) Quantitative analysis (staining intensity multiplied by area) of cTnT staining in (C). **P<0.01. ns, P>0.05.

(E) Quantitative analysis of cTnT staining in F⁺/P⁺ cells treated with basal differentiation medium (basal), 20 ng/ml BMP4, or 5 μM IWP2. **P<0.01.

(F) Relative expression of mesodermal- (upper panel) and cardiac progenitor cell- (lower panel) related genes during the generation of ieCPCs by quantitative PCR (qPCR). All gene expression levels were normalized to 2nd MEFs on day -2.

(G) qPCR analysis of markers of cardiovascular lineages (CMs, endothelial cells (ECs), and smooth muscle cells (SMCs)) and non-cardiovascular-mesodermal lineage (hematopoietic precursors, skeletal muscles, adipocytes, and chondrocytes) in F⁺/P⁺ ieCPCs that underwent cardiac-, endothelial-, or SMC-specific differentiation, or FBS-induced, non-specific differentiation for 10 days. Positive control cells were embryoid bodies (EBs) that differentiated from mouse embryonic stem cells (mESCs). EBs at differentiation day 6 were used to detect hematopoietic-precursor genes, and day 21 EBs were used for other lineages.

(H–K) Flow-cytometry analysis (H), immunofluorescence (I), Oil Red-O staining (J), and Alizarin Red-S staining (K) of the expression of non-cardiovascular-mesodermal lineage markers in ieCPCs cultured in the same differentiation conditions as in (G) and in the hematopoietic- (H), skeletal myogenic- (I), adipogenic- (J), and chondrogenic-specific (K) differentiation conditions. Mesodermal precursors derived from mESCs (mESC-mesoderm) served as positive controls for all non-cardiovascular-lineage differentiation. Data are means ± S.E.; Scale bars, 100 μm.

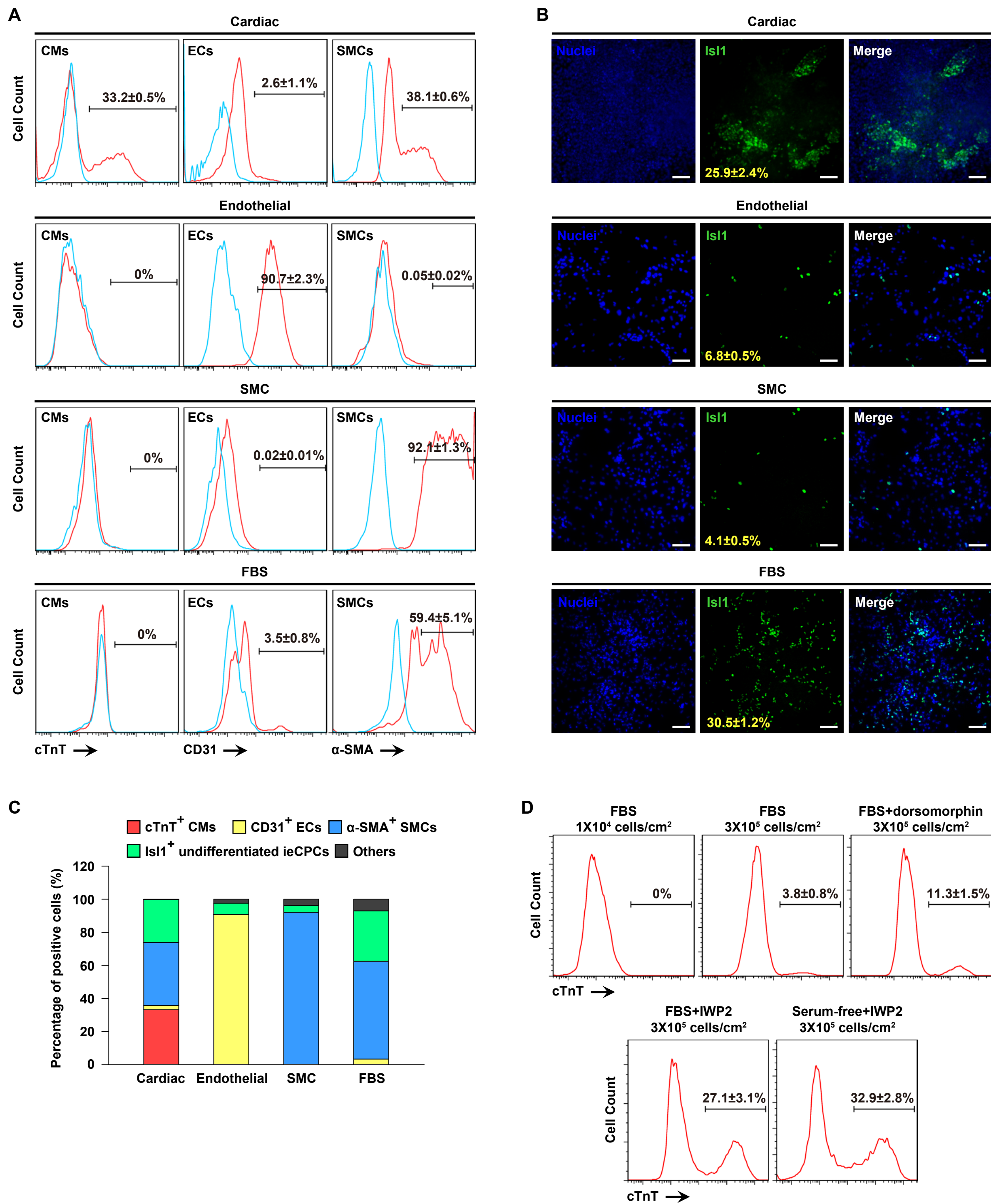


Figure S2. Differentiation of ieCPCs into Cardiovascular Lineages, Related to Figure 1.

(A) Flow-cytometry analysis of CM marker cTnT, EC marker CD31, and SMC marker α-SMA in ieCPCs that underwent cardiac-, endothelial-, or SMC-specific differentiation, or FBS-induced, non-specific differentiation for 10 days.

(B) Immunofluorescence analysis of the expression of the undifferentiated CPC marker Isl1 in ieCPCs cultured in the same differentiation conditions as in (A). Scale bars, 100 μm.

(C) Summary of (A) and (B) showing the cell-fate distribution in each differentiation condition.

(D) Flow-cytometry analysis of CM marker cTnT in ieCPCs cultured in FBS-containing or serum-free medium. Cells were differentiated at low ($1X10^4$ cells/cm^2) or high ($3X10^5$ cells/cm^2) seeding density with or without 2 μM BMP inhibitor dorsomorphin or 5 μM Wnt inhibitor IWP2.

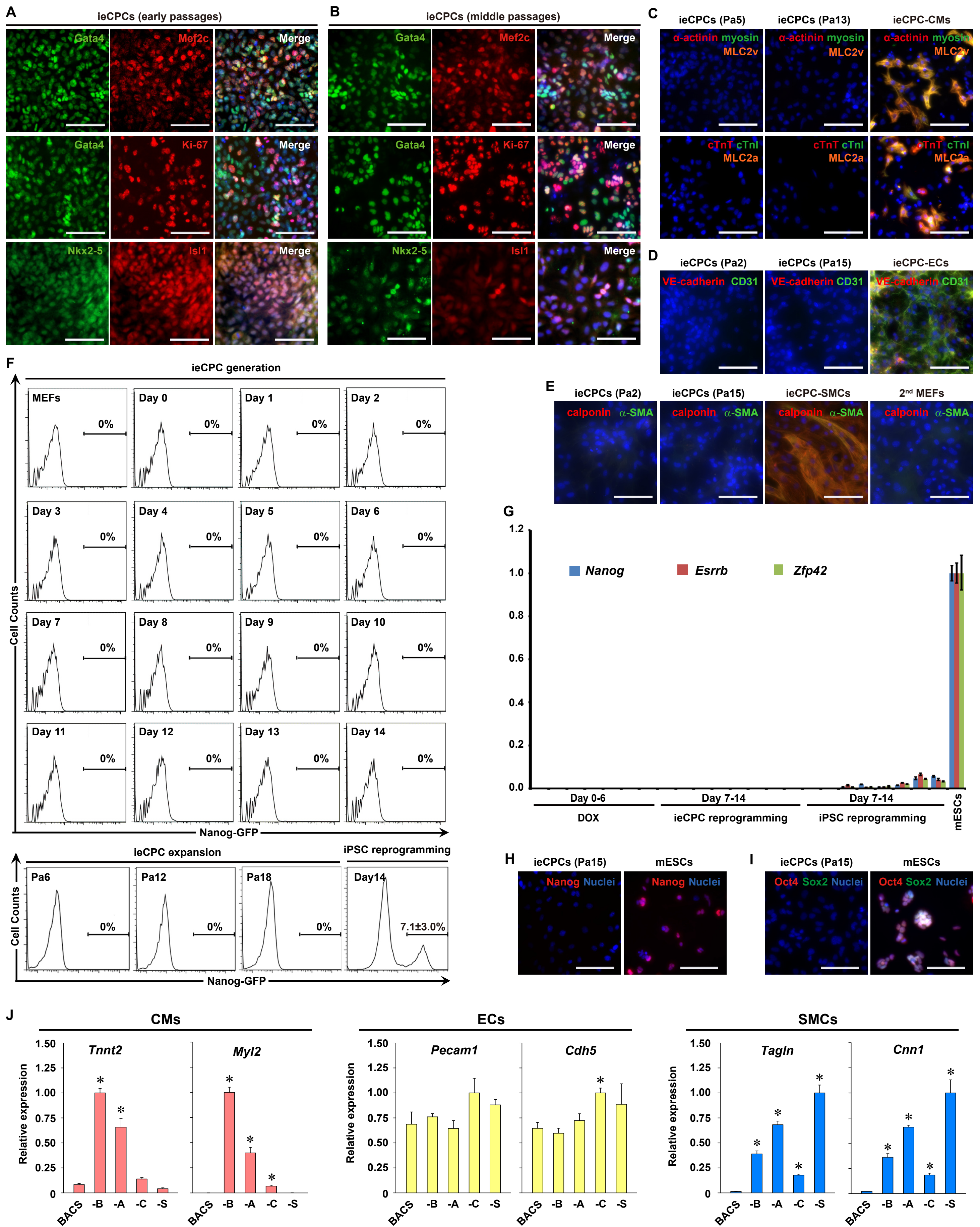


Figure S3. ieCPCs Express CPC But Not CM-, SMC-, EC-, or Pluripotency-Related Markers, Related to Figure 2.

(A–B) Immunofluorescence analyses of Gata4, Mef2c, Ki-67, Nkx2-5, and Isl1 in ieCPCs at early (<5) (A) and middle (5–10) (B) passages.

(C) Immunofluorescence analyses of CM-enriched markers, including α-actinin, myosin, MLC2v, cTnT, cTnI, and MLC2a in ieCPCs at early and late passages and CMs derived from ieCPCs (ieCPC-CMs). Pa, passage.

(D) Immunofluorescence analyses of the EC-enriched markers VE-cadherin and CD31 in ieCPCs at early and late passages and ECs derived from ieCPCs (ieCPC-ECs).

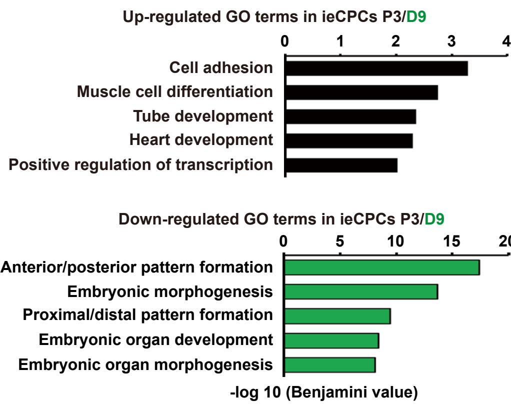
(E) Immunofluorescence analyses of the SMC-enriched markers calponin and α-SMA, in ieCPCs at early and late passages, SMCs derived from ieCPCs (ieCPC-SMCs), and 2nd MEFs.

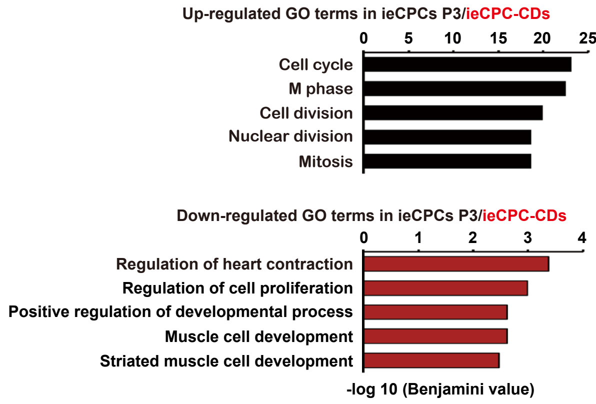
(F) Flow-cytometry analyses of Nanog-GFP$^+$ cells between day 0 and day 14 during the generation and long-term expansion of ieCPCs. Cells on day 14 of iPSC reprogramming were used as a positive control.

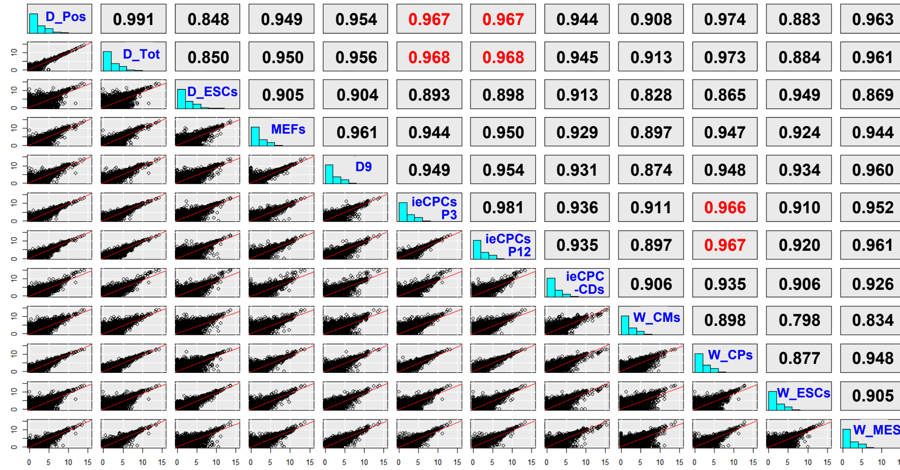
(G) qPCR analysis of *Nanog*, *Esrrb*, and *Zfp42* expression in cells treated with 2 μg/ml doxycycline (DOX) from day 0 to 6 and cells cultured in ieCPC- or iPSC-reprogramming conditions from day 7 to 14.

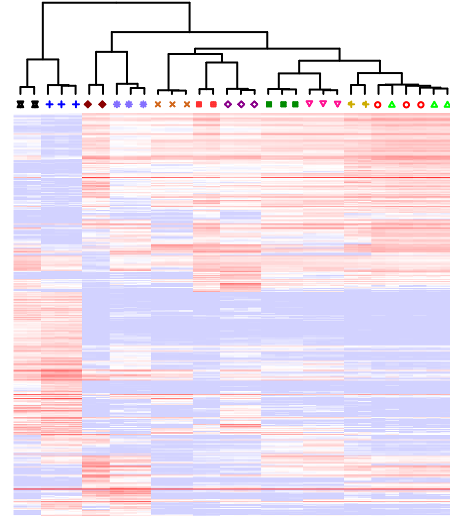
(H–I) Immunofluorescence analyses of pluripotent gene expression, including Nanog (H), Oct4, and Sox2 (I), in ieCPCs and mESCs.

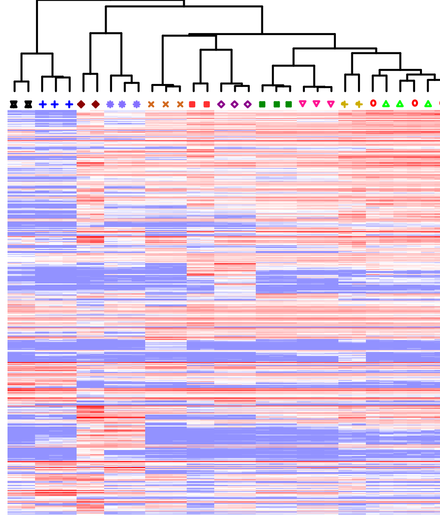
(J) Relative expression of the CM genes *Tnnt2* and *Myl2* , EC genes *Pecam1* and *Cdh5*, and SMC genes *Tagln* and *Cnn1* in ieCPCs cultured in BACS or after removal of individual compounds from BACS, as examined by qPCR. -, depleted; B, BMP4; A, Activin A; C, CHIR99021; and S, SU5402. Scale bars, 100 μm. Data are means ± S.E., $^*P<0.05$.

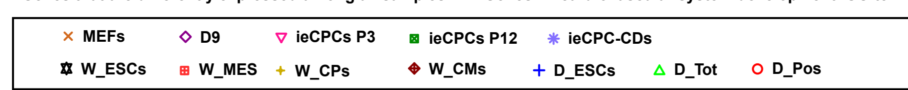

A
Up-regulated GO terms in ieCPCs P3/D9
0 1 2 3 4
Cell adhesion
Muscle cell differentiation
Tube development
Heart development
Positive regulation of transcription
Down-regulated GO terms in ieCPCs P3/D9
0 5 10 15 20
Anterior/posterior pattern formation
Embryonic morphogenesis
Proximal/distal pattern formation
Embryonic organ development
Embryonic organ morphogenesis
-log 10 (Benjamini value)


B
Up-regulated GO terms in ieCPCs P3/ieCPC-CDs
0 5 10 15 20 25
Cell cycle
M phase
Cell division
Nuclear division
Mitosis
Down-regulated GO terms in ieCPCs P3/ieCPC-CDs
0 1 2 3 4
Regulation of heart contraction
Regulation of cell proliferation
Positive regulation of developmental process
Muscle cell development
Striated muscle cell development
-log 10 (Benjamini value)


C
D_Pos 0.991 0.848 0.949 0.954 0.967 0.967 0.944 0.908 0.974 0.883 0.963
D_Tot 0.850 0.950 0.956 0.968 0.968 0.945 0.913 0.973 0.884 0.961
D_ESCs 0.905 0.904 0.893 0.898 0.913 0.828 0.865 0.949 0.869
MEFs 0.961 0.944 0.950 0.929 0.897 0.947 0.924 0.944
D9 0.949 0.954 0.931 0.874 0.948 0.934 0.960
ieCPCs P3 0.981 0.936 0.911 0.966 0.910 0.952
ieCPCs P12 0.935 0.897 0.967 0.920 0.961
ieCPC -CDs 0.906 0.935 0.906 0.926
W_CMs 0.898 0.798 0.834
W_CPs 0.877 0.948
W_ESCs 0.905
W_MES


D
Genes that are differently expressed among all samples


E
Genes in 'cardiovascular system development' GO term


MEFs
D9
ieCPCs P3
ieCPCs P12
ieCPC-CDs
W_ESCs
W_MES
W_CPs
W_CMs
D_ESCs
D_Tot
D_Pos

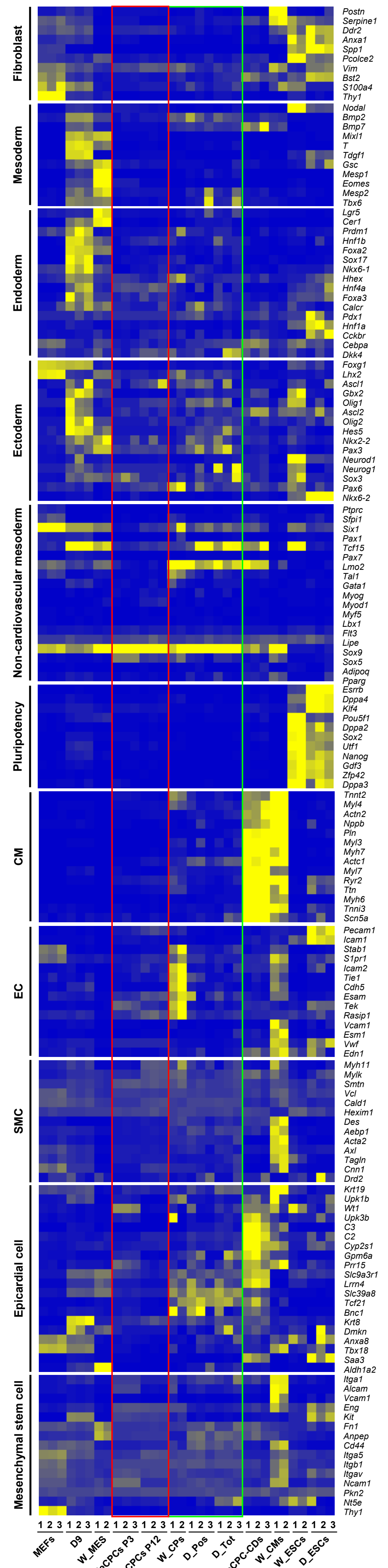

F
High
Low
Fibroblast
Postn
Serpine1
Ddr2
Anxa1
Spp1
Pcolce2
Vim
Bst2
S100a4
Thy1
Mesoderm
Nodal
Bmp2
Bmp7
Mixl1
T
Tdgf1
Gsc
Mesp1
Eomes
Mesp2
Tbx6
Endoderm
Lgr5
Cer1
Prdm1
Hnf1b
Foxa2
Sox17
Nkx6-1
Hhex
Hnf4a
Foxa3
Calcr
Pdx1
Hnf1a
Cckbr
Cebpa
Dkk4
Ectoderm
Foxg1
Lhx2
Ascl1
Gbx2
Olig1
Ascl2
Olig2
Hes5
Nkx2-2
Pax3
Neurod1
Neurog1
Sox3
Pax6
Nkx6-2
Non-cardiovascular mesoderm
Ptprc
Sfpi1
Six1
Pax1
Tcf15
Pax7
Lmo2
Tal1
Gata1
Myog
Myod1
Myf5
Lbx1
Flt3
Lipe
Sox9
Sox5
Adipoq
Pparg
Pluripotency
Esrrb
Dppa4
Klf4
Pou5f1
Dppa2
Sox2
Utf1
Nanog
Gdf3
Zfp42
Dppa3
CM
Tnnt2
Myl4
Actn2
Nppb
Pln
Myl3
Myh7
Actc1
Myl7
Ryr2
Ttn
Myh6
Tnni3
Scn5a
EC
Pecam1
Icam1
Stab1
S1pr1
Icam2
Tie1
Cdh5
Esam
Tek
Rasip1
Vcam1
Esm1
Vwf
Edn1
SMC
Myh11
Mylk
Smtn
Vcl
Cald1
Hexim1
Des
Aebp1
Acta2
Axl
Tagln
Cnn1
Drd2
Epicardial cell
Krt19
Upk1b
Wt1
Upk3b
C3
C2
Cyp2s1
Gpm6a
Prr15
Slc9a3r1
Lrrn4
Slc39a8
Tcf21
Bnc1
Krt8
Dmkn
Anxa8
Tbx18
Saa3
Aldh1a2
Mesenchymal stem cell
Itga1
Alcam
Vcam1
Eng
Kit
Fn1
Anpep
Cd44
Itga5
Itgb1
Itgav
Ncam1
Pkn2
Nt5e
Thy1
MEFs
D9
W_MES
ieCPCs P3
ieCPCs P12
W_CPs
D_Pos
D_Tot
ieCPC-CDs
W_CMs
W_ESCs
D_ESCs

Figure S4. Genome-Wide Transcriptional Analysis of ieCPCs, Related to Figure 3.

(A) Gene-ontology (GO) analyses of up-regulated and down-regulated genes in ieCPCs P3/cells at reprogramming day 9 (D9).

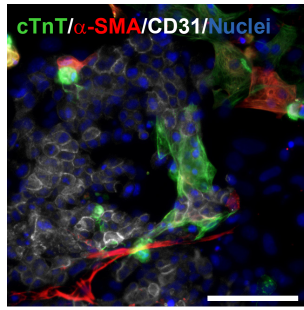
(B) GO analyses of up-regulated and down-regulated genes in ieCPCs P3/ieCPC cardiac derivatives (ieCPC-CDs).

(C) Pearson’s correlation analysis of the global gene-expression profile across all tested cell types. D_, Devine et al; W_, Wamstad et al.; Pos and Tot, CPCs with or without purification with a Smarcd3-GFP^{+} reporter; CPs, cardiac progenitors; MES, mesoderm.

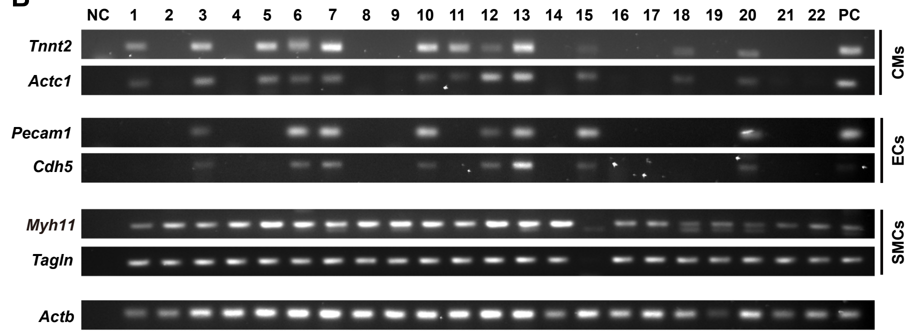
(D–E) Hierarchical clustering analysis of genes that are differently expressed (D) and that fall into the GO term of ‘cardiovascular system development’ (E) among all tested cell types. red, up-regulated; blue, down-regulated.

(F) Expression of marker genes of CPC-related lineages in all tested cell types detected by RNA-seq.

A



B



C

			Single-ieCPC-derived colonies																						Total (22)	%
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		
Lineage-specific markers	CM	*Tnnt2*	+		+		+	+	+			+	+	+	+		+			+		+			12	54.5
		Actc1	+		+		+	+	+			+	+	+	+		+			+		+				
	EC	*Pecam1*			+			+	+			+		+	+		+					+			8	36.4
		Cdh5			+			+	+			+		+	+		+					+				
	SMC	*Myh11*	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	21	95.5
		Tagln	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+		
Potency	CM + EC + SMC				+			+	+			+		+	+							+			7	31.8
	CM + EC																+								1	4.5
	CM + SMC		+				+						+							+					4	18.2
	SMC only			+		+				+	+					+		+	+		+		+	+	10	45.5

Figure S5. ieCPCs Exhibit Cardiovascular Potency in a Single-Cell-Derived Clone, Related to Figure 4 and Figure 5.

(A) Representative immunofluorescence analyses of CM marker cTnT, EC marker CD31, and SMC marker α-SMA expression in a clone derived from a single ieCPC after 28 days of differentiation. Scale bars, 100 μm.

(B) RT-PCR analysis of cardiovascular markers in colonies derived from single ieCPCs after 28 days of differentiation. Only clones positive for the housekeeping gene *Actb* are shown. NC, negative control, in which distilled water was used as templates; PC, positive control, in which ieCPC-CDs were used as templates.

(C) Summary of the cardiovascular-lineage potency of various ieCPC clones.

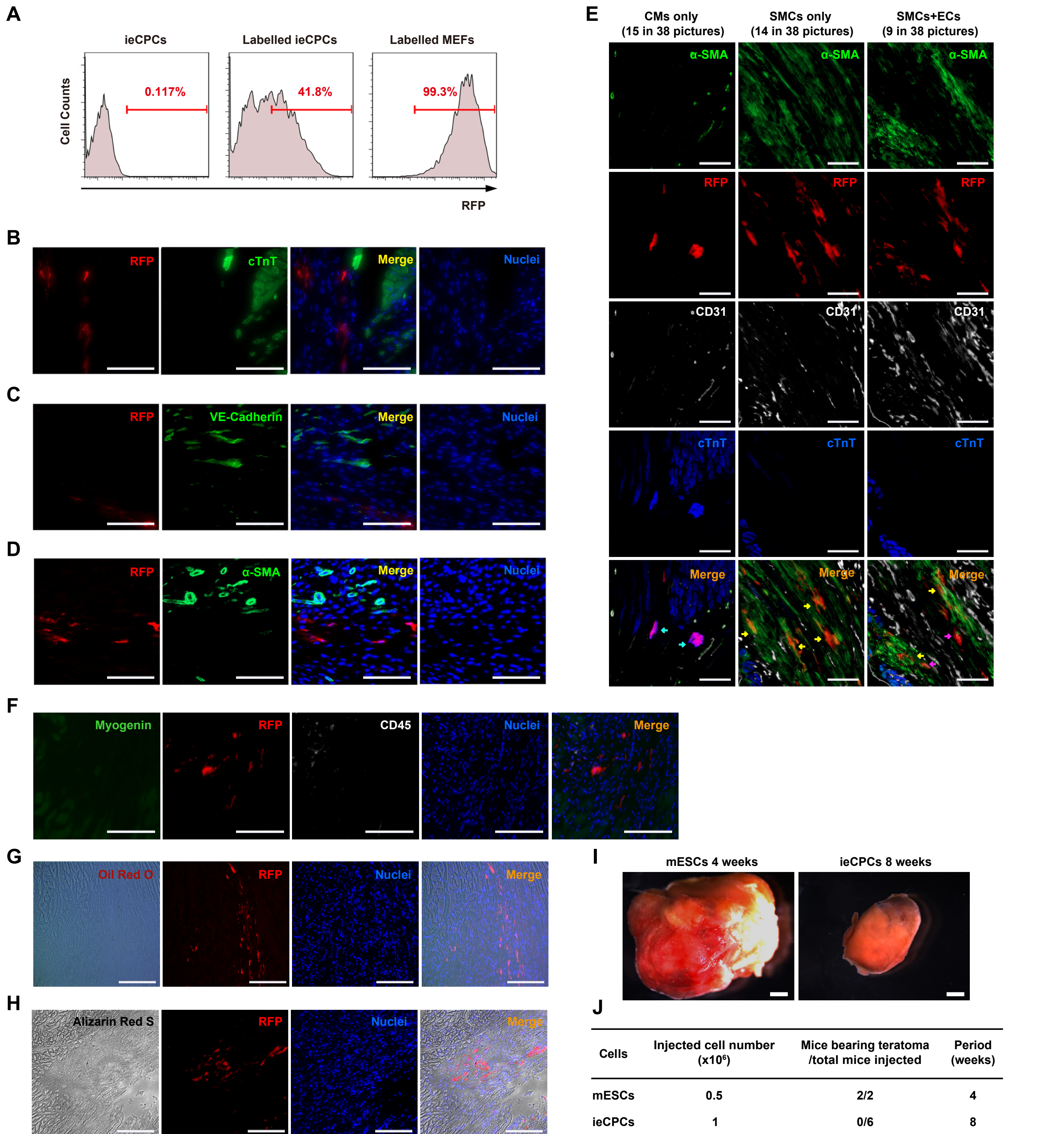


Figure S6. Transplantation of 2nd MEFs and ieCPCs *In Vivo*, Related to Figure 6.

(A) Flow-cytometry analyses of red fluorescent protein (RFP) in ieCPCs and 2nd MEFs before transplantation.

(B–D) Immunofluorescence analyses of RFP and CM (B), EC (C) and SMC (D) markers in tissue sections collected 2 weeks after transplanting RFP-labeled-2nd MEFs into infarcted hearts of immunodeficient mice. Scale bars, 100 µm.

(E) Immunofluorescence analyses of RFP and the CM marker cTnT, EC marker CD31, and SMC marker α-SMA in tissue sections collected 2 weeks after transplanting RFP-labeled ieCPCs at passage 10 into infarcted hearts of immunodeficient mice. Quantification of the percentage of different expression patterns is shown on top of each representative picture (n=38). Blue, yellow, and purple arrows indicate CM, SMC, and EC differentiation of ieCPCs, respectively. Scale bars, 100 µm.

(F) Immunofluorescence analyses of RFP and skeletal muscle marker myogenin and hematopoietic-precursor marker CD45 in tissue sections (n=51) collected 2 weeks after transplanting RFP-labeled ieCPCs at passage 10 into infarcted hearts of immunodeficient mice. Scale bars, 100 µm.

(G–H) Oil Red-O staining (G) and Alizarin Red-S (H) staining in similar tissue sections (n=51) in (F) containing engrafted, RFP-labeled ieCPCs. Scale bars, 100 µm.

(I) Representative images showing teratoma formation in infarcted mouse heart injected with mESCs (left panels) and lack of tumor formation in heart injected with ieCPCs (right panels). Scale bars, 1 mm.

(J) A summary of teratoma-forming ability of mESCs and ieCPCs.

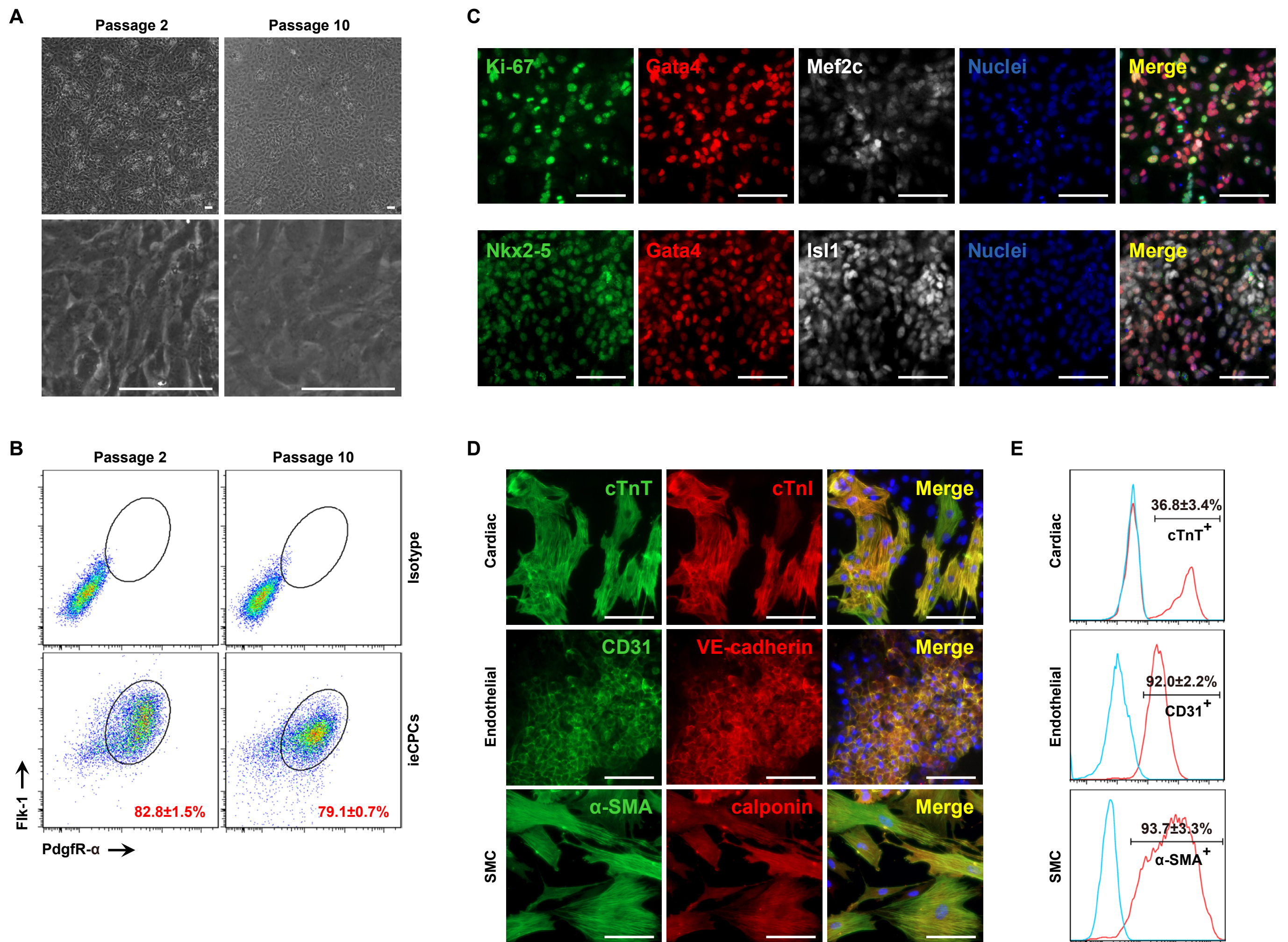


Figure S7. Reprogramming of Tail-Tip Fibroblasts into ieCPCs, Related to Figure 7.

(A) Representative images showing the typical morphology of ieCPCs derived from tail-tip fibroblasts (TTF-ieCPCs) at passages 2 and 10.

(B) Percentage of F^+/P^+ cells detected by flow cytometry at passages 2 and 10.

(C) Immunofluorescence analyses of Gata4, Mef2c, Ki-67, Nkx2-5, and Isl1 in TTF-ieCPCs at passage 10.

(D) Immunofluorescence analyses of the CM markers cTnT and cTnI, EC markers CD31 and VE-cadherin, and SMC markers α-SMA and calponin in TTF-ieCPCs cultured in CM-, EC-, and SMC-specific differentiation conditions for 10 days. Scale bars, 100 μm.

(E) Flow-cytometry analyses of the CM marker cTnT, EC marker CD31, and SMC marker α-SMA in TTF-ieCPCs cultured in the same differentiation conditions as in (D).

See also Movie S2.

SUPPLEMENTAL MOVIE LEGENDS

Movie S1. Beating Clusters Generated from 2nd MEF-Derived ieCPCs, Related to Figure 4

Spontaneously-contracting clusters observed after 2nd MEF-derived ieCPCs were cultured in cardiac differentiation conditions for 10 days.

Movie S2. Beating Clusters Generated from TTF-Derived ieCPCs, Related to Figure S7

Spontaneously-contracting clusters observed after TTF-derived ieCPCs were cultured in cardiac differentiation conditions for 10 days.

Movie S3. Beating Clusters Generated from mESC-Derived CPCs, Related to Figure 7

Spontaneously-contracting clusters observed after mESC-Derived CPCs were cultured in cardiac differentiation conditions for 10 days.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Preparation of Doxycyline-Inducible mouse 2nd MEFs and TTFs

2nd MEFs harboring doxycyline (DOX)-inducible transgenes encoding *Oct4*, *Sox2*, *Klf4*, and *c-Myc*, and a Nanog-GFP reporter to monitor the establishment of pluripotency were derived as previously described (Wernig et al., 2008). Heads, spinal cords, and developing organs were carefully removed from embryos. 2nd MEFs were cultured on gelatin-coated plates in MEF medium (high-glucose Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1X nonessential amino acids (NEAA)).

TTFs were prepared using established methods (Efe et al., 2011; Wang et al., 2014). Briefly, tail tips from neonatal and adult mice were minced with a sterile razor blade and then cultured in 10-cm culture dishes containing 2 ml MEF medium. After overnight culture, another 10 ml medium was added to the dish. Seven days later, fibroblasts that migrated out of the tissue samples were collected and expanded.

Cell-Activation and Signaling-Directed System-Based Cardiac Reprogramming

Cell-Activation and Signaling-Directed (CASD) system-based cardiac reprogramming was conducted as described previously (Efe et al., 2011). Briefly, 2nd MEFs at passage 3 were seeded onto geltrex-coated plates at a density of $2.5X10^4$ cells/well of a 12-well plate in MEF medium (day -2). DOX (2 µg/ml, Sigma-Aldrich) was added into the medium one day later (day -1) and cultured for another day. Medium was then changed to reprogramming medium (knockout DMEM supplemented with 5% knockout serum replacement (KSR), 15% embryonic stem cell (ESC)-qualified FBS, 1X Glutamax, 1X NEAA, 0.1 mM β-mercaptoethanol (β-ME), 1% ESC-qualified nucleosides (Millipore), 0.5 µM Jak inhibitor 1 (JI1; Millipore), and 2 µg/ml DOX) at day 0. This medium was renewed every 2 days. On day 6, medium was changed to transdifferentiation medium (knockout DMEM supplemented with 14% KSR, 1% ESC-qualified FBS, 1X Glutamax, 1X NEAA, 0.1 mM β-ME, and 1% ESC-qualified nucleosides, 3 µM CHIR99021 (Stemgent), and 0.5 µM JI1). On day 8, medium was switched to ieCPC basal medium (Advanced DMEM/F12: Neural basal (1:1) supplemented with 1X N2, 1X B27 without Vitamin A, 1X Glutamax, 1X NEAA, 0.05% bovine serum albumin (BSA), and 0.1 mM β-ME) supplemented with BACS (5 ng/ml BMP4, 10 ng/ml Activin A, 3 µM CHIR99021, and 2 µM SU5402 (Tocris Bioscience)). JI1 (0.5 µM) was included to suppress the establishment of pluripotency until the cells were ready for purification at day 13. This medium was renewed every 2 days.

To reprogram genetically unmodified TTFs, cells were infected with lentivirus harboring a DOX-inducible transgene encoding the reprogramming factors (Carey et al., 2009) for 12 hr as previously described (Wang et al., 2014). They were cultured in MEF medium for 3 days to recover from viral infection and then seeded onto 12-well geltrex-coated plates at a density of $2.5X10^4$ cells/well in MEF medium and reprogrammed as 2nd MEFs.

All cytokines are from a human source (R&D Systems), and all cultivation substances for cell cultures were from Life Technologies, unless stated otherwise.

Long-Term Expansion of ieCPCs

Flk-1$^+$/PdgfR-α$^+$ ieCPCs were purified on day 13 by fluorescence-activated cell sorting (FACS) and seeded onto 12-well geltrex-coated tissue culture plates at a density of $1X10^6$ cells/well in ieCPC basal medium with BACS. JI1 was

removed, and 250 μM ascorbic acid (Sigma-Aldrich) was added to promote cell growth. Confluent ieCPCs were split by incubating with collagenase B (Roche) at 37°C for 5 min, followed by accutase (Innovative Cell Technologies) at 37°C for another 2–5 min. ieCPCs were routinely passaged every four days by seeding onto 12-well plates at a density of $5X10^5$ cells/well, and medium was renewed every two days. Rock inhibitors, such as Y27632 (10 μM, Tocris Bioscience) and thiazovivin (1 μM, Cellagen Technology), can improve ieCPCs survival during sorting, passaging, or recovering from cryopreservation, but were not absolutely necessary.

Differentiation, Purification, and Expansion of CPCs Derived From mESCs

CPC differentiation of mESCs was performed as previously described (Kattman et al., 2011). Briefly, mESCs were dissociated, aggregated in ultralow attachment plates (Corning) at $7.5X10^4$ cells/ml, and cultured for two days in serum-free differentiation (SFD) medium (IMDM: F12 (3:1) supplemented with 0.5X N2, 0.5X B27 without Vitamin A, 1X Glutamax, 0.05% BSA, 450 μM MTG (Sigma-Aldrich), and 250 μM ascorbic acid). After 48 hr, embryoid bodies (EBs) were dissociated and reaggregated for another 24 hr with 0.5 ng/ml BMP4, 5 ng/ml Activin A, 5 ng/ml VEGF, and 250 μM ascorbic acid. $Flk\text{-}1^+/PdgfR\text{-}\alpha^+$ CPCs were then purified by FACS, seeded onto 12-well geltrex-coated tissue culture plates at a density of $1 X10^6$ cells/well in ieCPC basal medium with BACS and 250 μM ascorbic acid and routinely passaged every three days.

Differentiation of ieCPCs into Cardiovascular Lineages

For cardiac differentiation, ieCPCs were passaged onto 96-well matrigel-coated plates at a density of $3X10^5$ cells/cm^2 and cultured in SFD medium for 10 days. IWP2 (5 μM) was added to SFD medium during the first six days to increase the yield of cardiomyocytes (CMs) in the RNA-seq and functional characterization assays.

For smooth muscle cell (SMC), endothelial cell (EC), and FBS-induced non-specific differentiation, ieCPCs were seeded onto matrigel-coated plates at a density of $1X10^4$ cells/cm^2, unless stated otherwise. For EC differentiation, ieCPCs were cultured in Endothelial Cell Growth Medium-2 (EGM™-2; Lonza) for 10 days. For SMC differentiation, ieCPCs were cultured in SFD medium supplemented with TGF-β1 (2 ng/ml) and PDGF-BB (10 ng/ml) for 10 days. For FBS-induced differentiation, ieCPCs were cultured in SFD medium supplemented with 10% FBS for 10 days.

Positive control cells used in Figure S1G were differentiated from mESCs following the established protocol (Wobus et al., 2002). Briefly, mESCs were cultivated as EBs for 2 days in DMEM supplemented with 15% FBS using standard hanging-drop methods. Formed EBs were then transferred into ultralow attachment plates and cultured in suspension for 4 days. At day 7, floating EBs were plated onto gelatin-coated dishes and cultured for another two weeks.

Differentiation efficiency of each cell type was calculated with qPCR, flow cytometry, and/or an In Cell Analyzer 2000 (General Electric Healthcare).

Differentiation of ieCPCs and Mesoderm Precursors into Non-Cardiovascular Lineages

To induce hematopoietic differentiation, we adapted the second stage (hematopoietic specification) of an established protocol (Grigoriadis et al., 2010). Briefly, ieCPCs were seeded onto matrigel-coated plates at a density of $1X10^4$

cells/cm^2 and cultured in StemPro-34 medium containing VEGF (10 ng/ml), bFGF (1 ng/ml), IL-6 (10 ng/ml), IL-3 (40 ng/ml), IL-11 (5 ng/ml) and SCF (100 ng/ml) for 4 days.

For skeletal-muscle differentiation, we adapted the second stage of an established protocol (Mizuno et al., 2010). Briefly, ieCPCs were aggregated in ultralow attachment plates at 2.5X10^5 cells/ml in skeletal muscle–differentiation medium (DMEM supplemented with 5% horse serum, 1X Glutamax, 1X NEAA, and 0.1 mM β-ME) for 3 days of suspension culture. Cell aggregates were then dissociated, seeded onto matrigel-coated plates at a density of 1X10^4 cells/cm^2, and cultured in skeletal-muscle-differentiation medium for 10 days.

Adipogenic and chondrogenic differentiation were modified from previously described methods (Lee et al., 2004). Briefly, for adipogenic differentiation, ieCPCs were seeded onto matrigel-coated plates at a density of 1X10^4 cells/cm^2 and cultured in adipogenic-differentiation medium (α-MEM supplemented with 10% FBS, 1 μM dexamethasone (Sigma-Aldrich), 100 μg/ml 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 5 μg/ml insulin, and 60 μM indomethacin (Sigma-Aldrich)) for 14 days. The presence of adipocytes was monitored by Oil Red-O (Sigma-Aldrich) staining. For chondrogenic differentiation, 10 μl concentrated ieCPCs (3X10^6 cells/ml) suspended in chondrogenic-differentiation medium (α-MEM supplemented with 1% FBS, 50 μg/ml ascorbic acid, 6.25 μg/ml insulin, and 10 ng/ml TGF-β1) were seeded into the center of each well of 24-well plates and allowed to attach at 37°C for 2 hr. Chondrogenic-differentiation medium was then carefully added into the plates without detaching the cell aggregates. Cells were cultured in chondrogenic-differentiation medium for 2 weeks, dissociated, and replated onto matrigel-coated dishes for an additional week of culture before analysis. The presence of chondrocytes was monitored by Alizarin Red S (Sigma-Aldrich) staining.

For all non-cardiovascular-lineage differentiations, mesodermal precursors derived from E14 mESCs (harboring a Brachyury-GFP reporter that marks mesodermal precursors) were used as a positive control in parallel. Briefly, mESCs were cultivated as EBs for 2 days in DMEM supplemented with 20% FBS. Brachyury-GFP$^+$ cells were sorted by FACS and treated in the same conditions as ieCPCs in all experiments involving non-cardiovascular-lineage differentiation.

RT-PCR and Quantitative PCR

Total RNA was prepared using the RNeasy Plus Mini Kit with Qiashredder columns (Qiagen). On-column DNase digestion with RNase-free DNase (Qiagen) was performed to remove residual DNA. Total RNA (1 μg) was reverse transcribed into cDNA with the iScript cDNA Synthesis Kit (Bio-Rad). All quantitative PCR (qPCR) reactions were performed in triplicate with iQ SYBR Green Supermix (Bio-Rad) with one twentieth of a cDNA reaction per replicate, which was performed on an ABI 7900HT system (Invitrogen, Applied Biosystems). Expression data were analyzed with DataAssist V3.01 (Life Technologies). Each set of reactions was repeated with cDNA from at least three independent experiments. Primers are shown as followed (F, forward; R, Reverse; sequences were from 5' to 3')：*T*-F: TTGAACTTTCCTCCATGTGCTGA; *T*-R: TCCCAAGAGCCTGCCACTTT;

Mesp1-F: CATCGTTCCAGTACGCAGAA; *Mesp1*-R: CTAGAAGAGCCAGCATGTCG;

Mesp2-F: CCCAGAGCCTAGGAACAAGA; *Mesp2*-R: GGGTTCTGGAGACACAGAAAG;

Tbx6-F: CCGAGAAAATGGCAGAAACT; *Tbx6*-R: GTGTATCCCCACTCCCACAG;

Flk1-F: AAACCTCCTGCAAGCAAATG; *Flk1*-R: TCCAGAATCCTCTTCCATGC;

Pdgfra-F: AGAGGAGGAGCTTGAGGGAG; *Pdgfra*-R: AGAAAATCCGATACCCGGAG;
Gata4-F: CTGGAAGACACCCCAATCTC; *Gata4*-R: CAGGCATTGCACAGGTAGTG;
Mef2c-F: TGGAGAGATGAAGTGAAGCG; *Mef2c*-R: GCACAGCTCAGTTCCCAAAT;
Tbx5-F: GGCAGTGATGACCTGGAGTT; *Tbx5*-R: TGGTTGGAGGTGACTTTGTG;
Nkx2-5-F: CCAAGTGCTCTCCTGCTTTC; *Nkx2-5*-R: GGCTTTGTCCAGCTCCACT;
Isl1-F: TGTTTGAAATGTGCGGAGTG; *Isl1*-R: GCATTTGATCCCGTACAACC;
Actc1-F: AGCTGTCTTCCCGTCCATC; *Actc1*-R: GCTCTGGGCTTCATCACCTA;
Kcna5-F: ATGAGGCCCATCACTGTAGG; *Kcna5*-R: AAAATTGGAGACGATGACGG;
Pln-F: CCCAGCTAAGCTCCCATAAG; *Pln*-R: AACAGGCAGCCAAATGTGA;
Atp2a2-F: CTGGTGATATAGTGGAAATTGCTG; *Atp2a2*-R: GGTCAGGGACAGGGTCAGTA;
Slc8a1-F: TTGAGGACACCTGTGGAGTG; *Slc8a1*-R: TTCTCATACTCCTCGTCATCG;
Kcnj2-F: CCATGATCCTGTACCAGCAA; *Kcnj2*-R: AGAGATGGATGCTTCCGAGA;
Cacna1c-F: CCTGCTGGTGGTTAGCGTG; *Cacna1c*-R: TCTGCCTCCGTCTGTTTGGAA;
Scn5a-F: CTACCGCATAGTGGAGCACA; *Scn5a*-R: CGCTCCTCCAGGTAGATGTC;
Myh6-F: GCGCATTGAGTTCAAGAAGA; *Myh6*-R: CTTCATCCATGGCCAATTCT;
Myh7-F: AAGGGCCTGAATGAGGAGTAG; *Myh7*-R: TGCAAAGGCTCCAGGTCTGA;
Tnnt2-F: GTGTGCAGTCCCTGTTCAGA; *Tnnt2*-R: ACCCTCAGGCTCAGGTTCA;
Tnni3-F: GCCTCTGGAGATCATCATGG; *Tnni3*-R: CTCGGTAGTTGGCAGAGGAG;
Myl2-F: CTTCACTATCATGGACCAGAACAG; *Myl2*-R: ACACTTTGAATGCGTTGAGAATGGT;
Myl7-F: CTCACACTCTTCGGGGAGAA; *Myl7*-R: CTCTTCCTTGTTCACCACCC;
Gja5-F: CAGTTGAACAGCAGCCAGAG; *Gja5*-R: AGCTCCAGTCACCCATCTTG;
Ryr2-F: ACATCATGTTTTACCGCCTGAG; *Ryr2*-R: TTTGTGGTTATTGAACTCTGGCT;
Nanog-F: CCTCCAGCAGATGCAAGAACTC; *Nanog*-R: CTTCAACCACTGGTTTTTCTGCC;
Zfp42-F: TATGACTCACTTCCAGGGGG; *Zfp42*-R: AGAAGAAAGCAGGATCGCCT;
Esrrb-F: CTCGCCAACTCAGATTCGAT; *Esrrb*-R: AGAAGTGTTGCACGGCTTTG;
Pecam1-F: ACGCTGGTGCTCTATGCAAG; *Pecam1*-R: TCAGTTGCTGCCCATTCATCA;
Cdh5-F: CACTGCTTTGGGAGCCTTC; *Cdh5*-R: GGGGCAGCGATTCATTTTTCT;
Tek-F: GAGTCAGCTTGCTCCTTTATGG; *Tek*-R: AGACACAAGAGGTAGGGAATTGA;
Tagln-F: CAACAAGGGTCCATCCTACGG; *Tagln*-R: ATCTGGGCGGCCTACATCA;
Myh11-F: AAGCTGCGGCTAGAGGTCA; *Myh11*-R: CCCTCCCTTTGATGGCTGAG;
Cnn1-F: TCTGCACATTTTAACCGAGGTC; *Cnn1*-R: GCCAGCTTGTTCTTTACTTCAGC;
Spi1-F: ATGTTACAGGCGTGCAAAATGG; *Spi1*-R: TGATCGCTATGGCTTTCTCCA;
Gata1-F: TGGGGACCTCAGAACCCTTG; *Gata1*-R: GGCTGCATTTGGGGAAGTG;
Runx1-F: GATGGCACTCTGGTCACCG; *Runx1*-R: GCCGCTCGGAAAAGGACAA;
Myod1-F: CCACTCCGGGACATAGACTTG; *Myod1*-R: AAAAGCGCAGGTCTGGTGAG;
Myog-F: GAGACATCCCCCTATTTCTACCA; *Myog*-R: GCTCAGTCCGCTCATAGCC;
Myf5-F: AAGGCTCCTGTATCCCCTCAC; *Myf5*-R: TGACCTTCTTCAGGCGTCTAC;

Adipoq-F: TGTTCCTCTTAATCCTGCCCA; *Adipoq*-R: CCAACCTGCACAAGTTCCCTT;
Pparg-F: TCGCTGATGCACTGCCTATG; Pparg-R: GAGAGGTCCACAGAGCTGATT;
Lipe-F: CCAGCCTGAGGGCTTACTG; *Lipe*-R: CTCCATTGACTGTGACATCTCG;
Sox5-F: AGCCGCAATGCAGGTTTCT; *Sox5*-R TTGTGCTCTTGTCTGTGTGAAT;
Sox9-F: GAGCCGGATCTGAAGAGGGA; *Sox9*-R: GCTTGACGTGTGGCTTGTTC;
Acan-F: CCTGCTACTTCATCGACCCC; *Acan*-R: AGATGCTGTTGACTCGAACCT;
Actb-F: TTCTTTGCAGCTCCTTCGTT; *Actb*-R: ATGGAGGGGAATACAGCCC;
Rpl7-F: TCTCTCTTCTTTTCCGGCTG; *Rpl7*-R: TTCTTGAGGGTTTCTGGCAC

For RT-PCR analyses of clonal assays, total RNA of a single ieCPC clone was collected and reverse-transcribed using the Power SYBR® Green Cells-to-Ct™ Kit (Life Technologies). PCR reactions were performed using the Taq PCR Kit (New England Biolabs). The house-keeping gene *Actb* was used as an internal control.

Immunofluorescence Staining Analysis

To analyze intracellular markers, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 15 min, washed three times with phosphate buffered saline (PBS), and incubated in PBS containing 0.3% Triton X-100 (Sigma-Aldrich) and 3% IgG-free BSA (Jackson ImmunoResearch) for 1 hr at room temperature. To analyze cell-surface markers, such as CD31 and VE-Cadherin, cells were grown on matrigel-coated glass coverslips (Warner Instruments) and fixed with pre-chilled acetone for 2 min, rehydrated, washed three times with PBS, and incubated in PBS containing 3% IgG-free BSA for 1 hr at room temperature. Primary antibodies were incubated overnight at 4ºC and included Gata4 (sc-25310, Santa Cruz Biotechnology; 1:200); Mef2c (5030, Cell Signaling Technology; 1:200); Isl1 (39.4D5, Developmental Studies Hybridoma Bank; 1:50); Nkx2-5 (ab35842, Abcam; 1:50); Ki-67 (550609, BD Biosciences; 1:200); cardiac troponin T (cTnT) (MS-295-P1, Thermo Scientific; 1:400); myosin (MF20, Developmental Studies Hybridoma Bank; 1:400); α-actinin (A7732, Sigma-Aldrich; 1:400); myosin light chain (MLC) 2v (10906-1-AP, ProteinTech Group; 1:200); MLC2a (310 11, Synaptic Systems; 1:400); myosin heavy chain (MHC) (ab15, Abcam; 1:500); cardiac troponin I (cTnI) (SC-15368, Santa Cruz Biotechnology; 1:200); α-smooth muscle actin (α-SMA) (A-2547, Sigma-Aldrich; 1:800); calponin (C-2687, Sigma-Aldrich; 1:800); cluster of differentiation 31 (CD31) (BD-550274, BD Biosciences; 1:50); vascular endothelial (VE-)cadherin (SC-9989, Santa Cruz Biotechnology; 1:50); myogenin (SC-12732, Santa Cruz Biotechnology; 1:200); Oct4 (SC-5279, Santa Cruz Biotechnology; 1:500); Nanog (ab80892, Abcam; 1:500); Sox2 (AB5603, EMD Millipore; 1:500), and CD45 (BD-550539, BD Biosciences; 1:50). After washing cells three times with PBS, they were incubated with isotype-matched Alexa Fluorescence-conjugated secondary antibodies (Invitrogen) for 1 hr at room temperature. Cell nuclei were stained with DAPI (D9542, Sigma-Aldrich). Images were acquired with a Zeiss Axio Observer microscope equipped with an Axiocam HRm camera and processed with Axiovision 4.7.1 software. The expression of cTnT (measured by multiplying staining intensity and area) and Isl1 or α-SMA (percentage in total cells) were monitored and analyzed by an IN Cell Analyzer 2000.

For immunostaining in cell-transplantation studies, heart samples were collected two weeks after transplantation, fixed in 0.4% paraformaldehyde overnight, dehydrated in 20% sucrose (Sigma-Aldrich), embedded

in OCT compound (VWR International), and frozen in dry ice–conditioned isopentane. Heart samples were cut vertically in 8-µm sections and stained as described above.

Flow Cytometry and Sorting

To detect cell-surface markers, dissociated cells were incubated with antibodies including Phycoerythrin (PE)-conjugated Flk-1 (12-5821, eBioscience; 1:50), Allophycocyanin (APC)-conjugated-PdgfR-α (17-1401, eBioscience; 1:100), PE-conjugated c-Kit (BD-553869, BD Biosciences; 1:100), APC-conjugated CD45 (BD-559864, BD Biosciences; 1:100), or APC-conjugated CD31 (17-0311, eBioscience; 1:100) antibodies at room temperature for 1–1.5 hr. Isotype-matched normal IgGs were used as negative controls.

To detect intracellular antigens, dissociated cells were fixed and permeabilized with a BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences), and then incubated with antibodies for cTnT (MS-295-P1, Thermo Scientific; 1:200) and α-SMA (A-2547, Sigma-Aldrich; 1:200) at 4°C overnight. After three washes, cells were incubated with isotype-matched Alexa Fluorescence-conjugated secondary antibodies (Invitrogen) for 1 hr at room temperature and detected by an LSR II Flow Cytometer (BD Biosciences).

For sorting, dissociated cells were incubated with PE-conjugated Flk-1 antibody (1:25) and APC-conjugated PdgfR-α antibody (1:50) for 2 hr at 4°C with rotation. Flk-1^+/PdgfR-α^+ cells were sorted with an Aria III Cell Sorter (BD Biosciences). Isotype-matched normal IgGs served as negative controls.

RNA Sequencing and Alignment

Total RNA was prepared using the RNeasy Plus Mini Kit with Qiashredder columns (Qiagen). Potentially residual DNA was removed by on-column DNase digestion with RNase-free DNase (Qiagen). Total RNA (10 µg) was used as input material for preparing the RNA sequencing (RNA-seq) libraries with an Ovation Ultralow System V2 (NuGEN). Amplified libraries were sequenced on HiSeq 2500.

Paired-end RNA-Seq reads were aligned to the reference assembly mm9 with Tophat 2.0.13 (Kim et al., 2013). Aligned reads were assigned to genes using "featureCounts" (Liao et al., 2014), part of the Subread suite (http://subread.sourceforge.net/). Differential expression P-values were calculated using edgeR (Robinson et al., 2010), an R package available through Bioconductor. Genes without at least two samples with a CPM (counts per million) value between 0.5 and 5000 were filtered out. Remaining expression values were normalized using "calcNormFactors" in edgeR. P-values for expression differences between samples were then calculated in edgeR with a negative binomial distribution for gene expression. Finally, FDR (false discovery rate) for each P-value was calculated by the built-in R function "p.adjust" using the Benjamini-Hochberg method. For hierarchical cluster analyses, Cluster 3.0 software was used. For gene-ontology analyses of gene-set enrichment among different samples, the DAVID Functional Annotation Tool (http://david.abcc.ncifcrf.gov/tools.jsp) was used.

Samples in this work and two previous studies (Devine et al., 2014; Wamstad et al., 2012) were normalized using the RUVseq (Risso et al., 2014) bioconductor package in R. Genes with five or less raw counts in, at most, two samples were filtered out from the analyses. From the remaining set of genes, coefficients of variation were computed for each gene across all samples within each study. One-thousand genes with the lowest mean coefficient of variation of expression across the three studies were then chosen as control genes. Two factors (k=2) were

supplied to the RUVg function to remove the unwanted variation associated with the lab of origin for each sample. plotPCA function was used to create the principal component–analyses plot in Figure 3C.

Transmission Electron Microscopy

Transmission electron microscopy was performed as described (Fu et al., 2013). Briefly, cells were fixed in 2% glutaraldehyde and 1% paraformaldehyde in 0.1M sodium cacodylate buffer, pH 7.4, post-fixed in 2% osmium tetroxide in the same buffer, en-block stained with 2% aqueous uranyl acetate, dehydrated in acetone, infiltrated, and embedded in LX-112 resin (Ladd Research Industries, Burlington, VT). Samples were ultrathin sectioned on a Reichert Ultracut S ultramicrotome and counter stained with 0.8% lead citrate. Grids were examined on a JEOL JEM-1230 transmission electron microscope (JEOL USA, Inc.) and photographed with the GatanUltrascan 1000 digital camera (Gatan Inc.).

Electrophysiology and Intracellular Ca^{2+} Measurements

Contracting cardiomyocytes derived from ieCPCs (ieCPC-CMs) were dispersed by first treating cells with Collagenase B for 5-10 min and then with Accutase for 5 min. Dissociated cells were replated onto matrigel-coated coverslips (Warner Instruments). After visible beating was reconfirmed, the coverslips were loaded with the Ca^{2+}-sensitive fluorescent indicator Fluo-4 (see below), and then placed in a superfusion bath (Warner Instruments) on a Nikon TiS inverted microscope equipped with a microfluorometer (IonOptix). Superfusion solutions were warmed to 30°C with a superfusion system and heated perfusion pencil (ValveLink, AutoMate Scientific). Single spontaneously contracting myocytes and small clusters were selected for study, with one cell under amphotericin B–perforated patch clamp (Spencer et al. 2014). Briefly, patch electrodes of approximately 2 MΩ (WPI) were tip-filled by dipping (20 s) in an intracellular solution containing (mM): KCl 120, NaHEPES 20, MgATP 10, K_2EGTA 5, $MgCl_2$ 2, adjusted to pH 7.1 with KOH, and then back filled with the same solution containing amphotericin B (240 µg/ml). Coverslips were superfused at a constant flow (Warner Instruments) with modified Tyrode's extracellular solution containing (mM): NaCl 137, NaHEPES 10, dextrose 10, KCl 5, $CaCl_2$ 2, and $MgCl_2$ 1, set to pH 7.4 with NaOH. Spontaneous action potentials (APs) were detected in current-clamp mode with zero applied current and digitized for 30 s per data file.

The ieCPC-CMs on coverslips were loaded with Fluo-4 AM in a 1:10 mixture of the indicator dissolved in dry dimethyl sulfoxide at 5 mM, plus Powerload concentrate (Life Technologies). This mixture was diluted 100-fold into extracellular Tyrode's solution and substituted for culture medium in dishes containing coverslips (final indicator concentration, 5 µM). Cells were loaded with dye for 20 min at room temperature and placed in dye-free extracellular solution for 20 min to allow for indicator de-esterification before recordings were taken. Fluo-4 fluorescence transients were recorded with a standard filter set with excitation and emission centered on 480 nm and 535 nm, respectively (Chroma Technology).

Fluorescence was obtained from contracting cells plus a cell-free border using a cell-framing adaptor (IonOptix). Background fluorescence, recorded after removing the cell(s) from the field of view, was subtracted from all recordings. Between sampling periods, the excitation light was blocked with a shutter (Vincent Associates). Background-corrected fluorescence transients were expressed relative to the diastolic fluorescence between

spontaneous APs and were considered to reflect underlying changes in intracellular Ca^{2+} concentration. Unless stated, reagents were from Sigma Chem. Co. Pharmacological studies were conducted on the spontaneous fluorescence transients of non-patch-clamped myocytes, and, to maximize coverslip usage, pharmacological agents were applied locally (only to the cell or cluster of interest) with the perfusion pencil.

APs and fluorescence transients were digitized at 5 kHz and low-pass filtered at 2 kHz. The maximum upstroke velocity of the AP (V_{max}) was calculated with pClamp software (Molecular Devices). AP durations, determined from V_{max}, were calculated for every AP in a given data file using in-house analysis routines implemented in Excel 2007 (Microsoft). Voltages were corrected for a –5.6 mV liquid junction potential.

Functional Assays of Endothelial Cells *In Vitro*

To analyze tube formation on matrigel *in vitro*, cells were seeded on top of a thin layer of matrigel at a density of $2.5X10^4$ cells/well of a 12-well plate. After 24 hr, cells were subjected to brightfield imaging microscopy to visualize formation of tube-like structures. Uptake of acetylated low-density lipoprotein (ac-LDL) was assessed by incubating cells with 5 µg/ml of ac-LDL conjugated with Alexa Fluor-594 (Invitrogen) for 4 hr. After incubation, cells were washed with PBS before fluorescence microscopy.

Contractile Assay of Smooth Muscle Cells *In Vitro*

ieCPCs, ieCPC-SMCs, and primary SMCs were treated with carbachol (100 µM, Sigma-Aldrich) and monitored with a Zeiss Axio Observer microscope in a time-lapse manner at 10-minute intervals for 1 hr. Cell-surface areas were then calculated with ImageJ software.

Clonal Assay

Single ieCPCs were isolated by FACS, plated onto 384-well plates at one cell per well, and cultured for 3 days in SFD medium supplemented with 20% FBS; 1 µM thiazovivin (Stemgent) was added into the culture medium to improve ieCPCs survival. After overnight culture, each well of the plate was monitored with a microscope and rare wells that contained more than one cell were excluded from experiments. At day 4, medium was changed to SFD supplemented with 10 ng/ml FGF2, 100 ng/ml IGF1, 10 ng/ml EGF, and 250 µM ascorbic acid to promote cell growth. Colonies were scored after 28 days of culture.

***In Vivo* Transplantation**

Myocardial infarction was induced by permanently ligating the left anterior descending artery in 10–12-week-old male NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice (The Jackson Laboratory) as described (Qian et al., 2012). ieCPCs and their parental 2nd MEFs were labeled by retrovirus harboring an RFP reporter and transplanted into infarcted hearts. One million donor cells were injected along the boundary between the infarct and border zones immediately after coronary ligation. For teratoma-formation experiments, mESCs (0.5 million cells, n=2) and ieCPCs (1 million cells, n=6) were similarly injected into the infarcted hearts of NSG immunodeficient mice that were 10–12-weeks-old. All mouse work was conducted according to the *Guide for the Care and Use of Laboratory Animals*, as adopted by the National Institutes of Health, and with approval of the University of California, San Francisco Institutional Animal Care and Use Committee.

Echocardiography

Echocardiography was performed with the Vevo 770 High-Resolution Micro-Imaging System (FUJIFILM VisualSonics) with a 15-MHz linear-array ultrasound transducer as described previously (Qian et al., 2012). Briefly, M-mode tracing with a sweep speed of 50 mms^{-1} at the papillary muscle level was used to measure left-ventricular anterior and posterior wall thickness. These data were then used for calculating the shortening fraction. B-mode was used for two-dimensional measurements of end-systolic and end-diastolic dimensions, which were obtained for calculating the ejection fraction. All surgeries, cell transplantation, and subsequent analyses were performed blinded and decoded only at the end of the experiments.

Determination of Scar Size

At the end of the experiments, mice were anaesthetized by 5% isoflurane, and 0.1M KCl was injected into mouse hearts to stop them at diastole. The hearts were then fixed, cut vertically in 5-µm sections, and further processed for histology analyses.

To determine the scar size, Masson-Trichrome staining was performed following a standard protocol (Qian et al., 2012) on hearts 12 weeks after coronary artery ligation. For each group, three representative hearts that had a shortening fraction similar to the average value of the group were collected. Then the scar size was measured with ImagePro software to calculate the percentage of the total left-ventricular area (red plus blue) showing fibrosis (blue). For each sample, scar size was evaluated on transverse sections spanning eight levels and calculated as previously described (Takagawa et al., 2007). From each level, we measured two slices of tissues as technical repeats (for a total of 48 sections).

Statistical Analysis

Data were presented as means ± S.E.. Statistical significance of differences was estimated by student's *t*-test in Microsoft Excel. Differences were considered significant when $P\text{-value}<0.05$.

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