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Supplemental Information

Cell-Surface Marker Signature for Enrichment of Ventricular Cardiomyocytes Derived from Human Embryonic Stem Cells

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Supplemental Information

Figure S1.

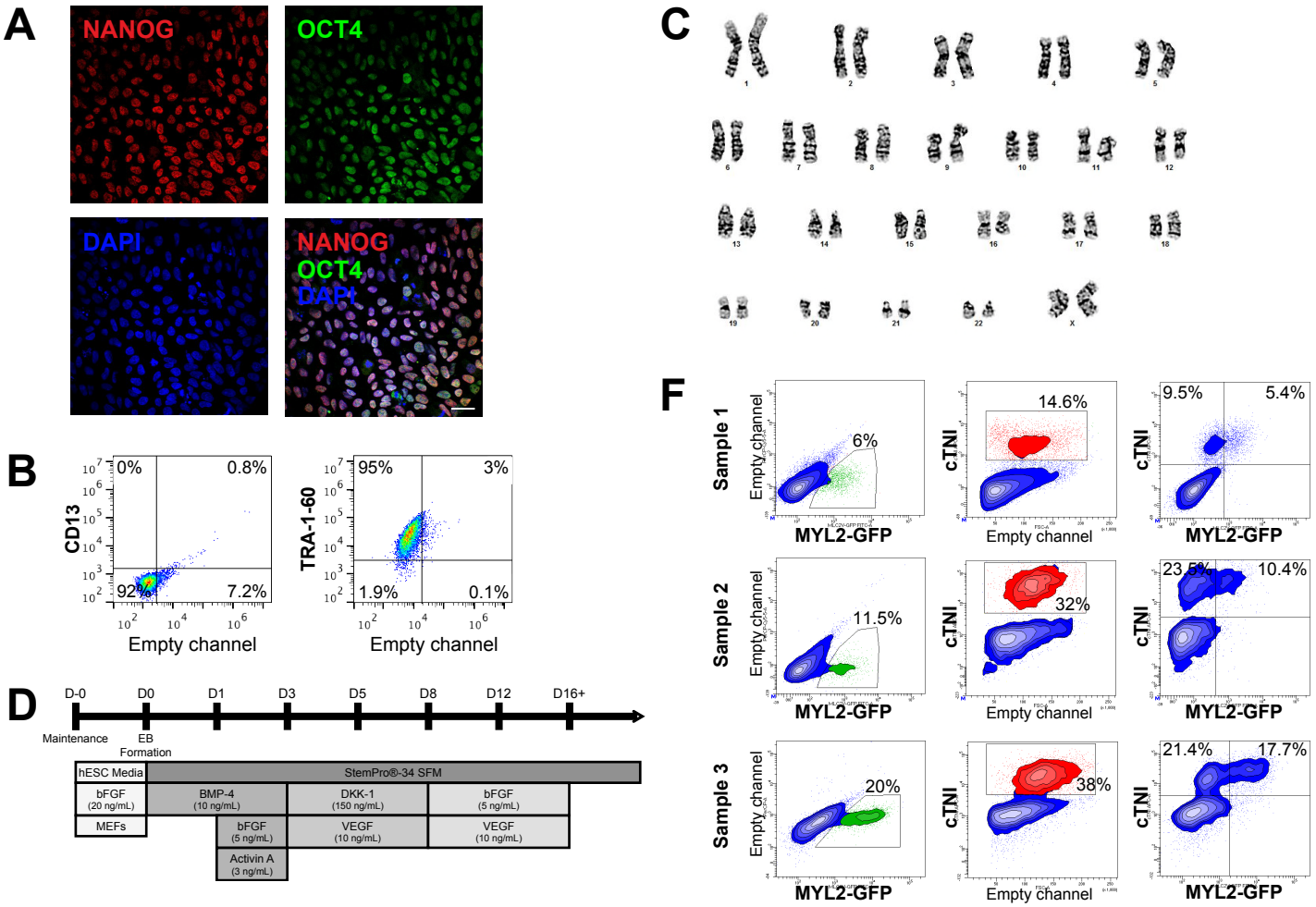

A
NANOG
OCT4
DAPI
NANOG
OCT4
DAPI
B
CD13
0%
0.8%
92%
7.2%
Empty channel
TRA-1-60
95%
3%
1.9%
0.1%
Empty channel
C
D
D-0
D0
D1
D3
D5
D8
D12
D16+
Maintenance
EB Formation
hESC Media
StemPro®-34 SFM
bFGF (20 ng/mL)
BMP-4 (10 ng/mL)
DKK-1 (150 ng/mL)
bFGF (5 ng/mL)
MEFs
bFGF (5 ng/mL)
VEGF (10 ng/mL)
VEGF (10 ng/mL)
Activin A (3 ng/mL)
F
Sample 1
Sample 2
Sample 3
Empty channel
MYL2-GFP
6%
14.6%
9.5%
5.4%
11.5%
32%
23.5%
10.4%
20%
38%
21.4%
17.7%
cTNI
E
Fetal atria
Fetal ventricle
Adult atria
Adult ventricle
Relative expression
T
ISL1
NKX2-5
TNNI3
TNNT2
MYL7
MYL2
Time (days of differentiation)

Figure S2.

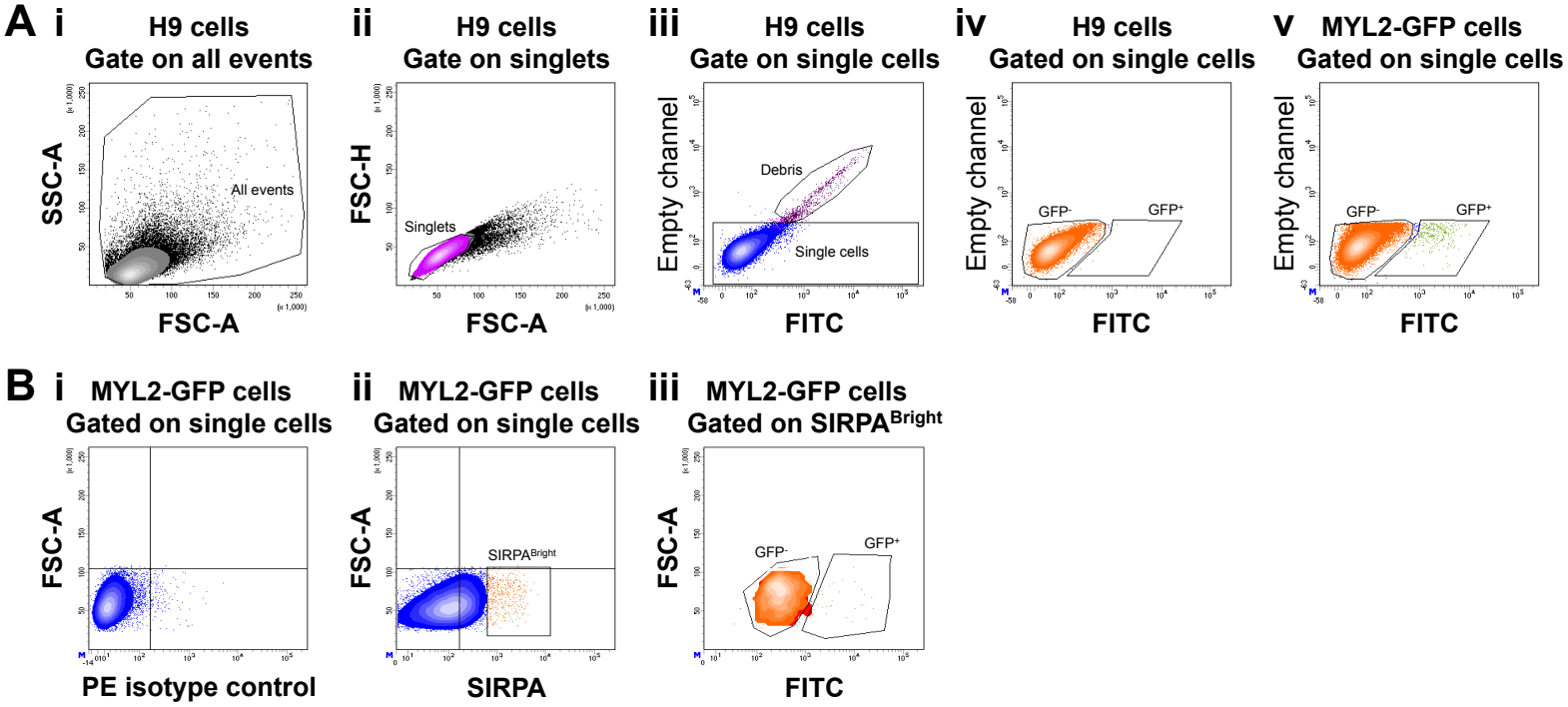
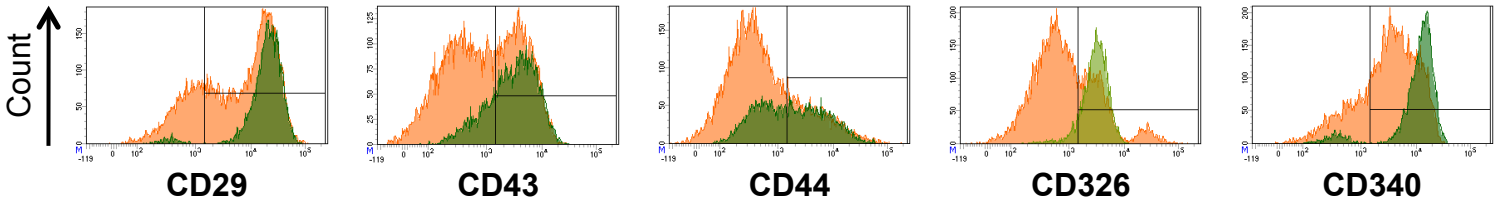

A
i
H9 cells
Gate on all events
SSC-A
FSC-A
All events
ii
H9 cells
Gate on singlets
FSC-H
FSC-A
Singlets
iii
H9 cells
Gate on single cells
Empty channel
FITC
Debris
Single cells
iv
H9 cells
Gated on single cells
Empty channel
FITC
GFP-
GFP+
v
MYL2-GFP cells
Gated on single cells
Empty channel
FITC
GFP-
GFP+
B
i
MYL2-GFP cells
Gated on single cells
FSC-A
PE isotype control
ii
MYL2-GFP cells
Gated on single cells
FSC-A
SIRPA
SIRPA Bright
iii
MYL2-GFP cells
Gated on SIRPA Bright
FSC-A
FITC
GFP-
GFP+

Figure S3.



CD29

MFI ratio	1.63
GFP+	94.7%
GFP-	77.9%

CD43

MFI ratio	1.81
GFP+	78.8%
GFP-	47.2%

CD44

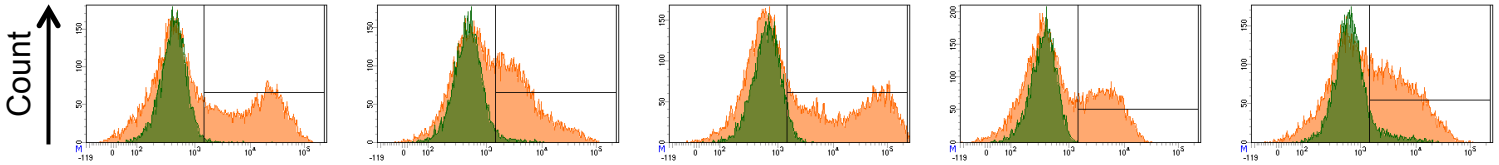
MFI ratio	2.15
GFP+	57.3%
GFP-	29.6%

CD326

MFI ratio	2.13
GFP+	90.3%
GFP-	42.8%

CD340

MFI ratio	2.02
GFP+	90.6%
GFP-	78.6%



CD49a

MFI ratio	0.39
GFP+	7.8%
GFP-	43.2%

CD49b

MFI ratio	0.32
GFP+	13.3%
GFP-	48.3%

CD90

MFI ratio	0.37
GFP+	18.5%
GFP-	50.7%

CD140b

MFI ratio	0.46
GFP+	5.6%
GFP-	35.8%

CD195

MFI ratio	0.36
GFP+	22.8%
GFP-	51.7%

Figure S4.

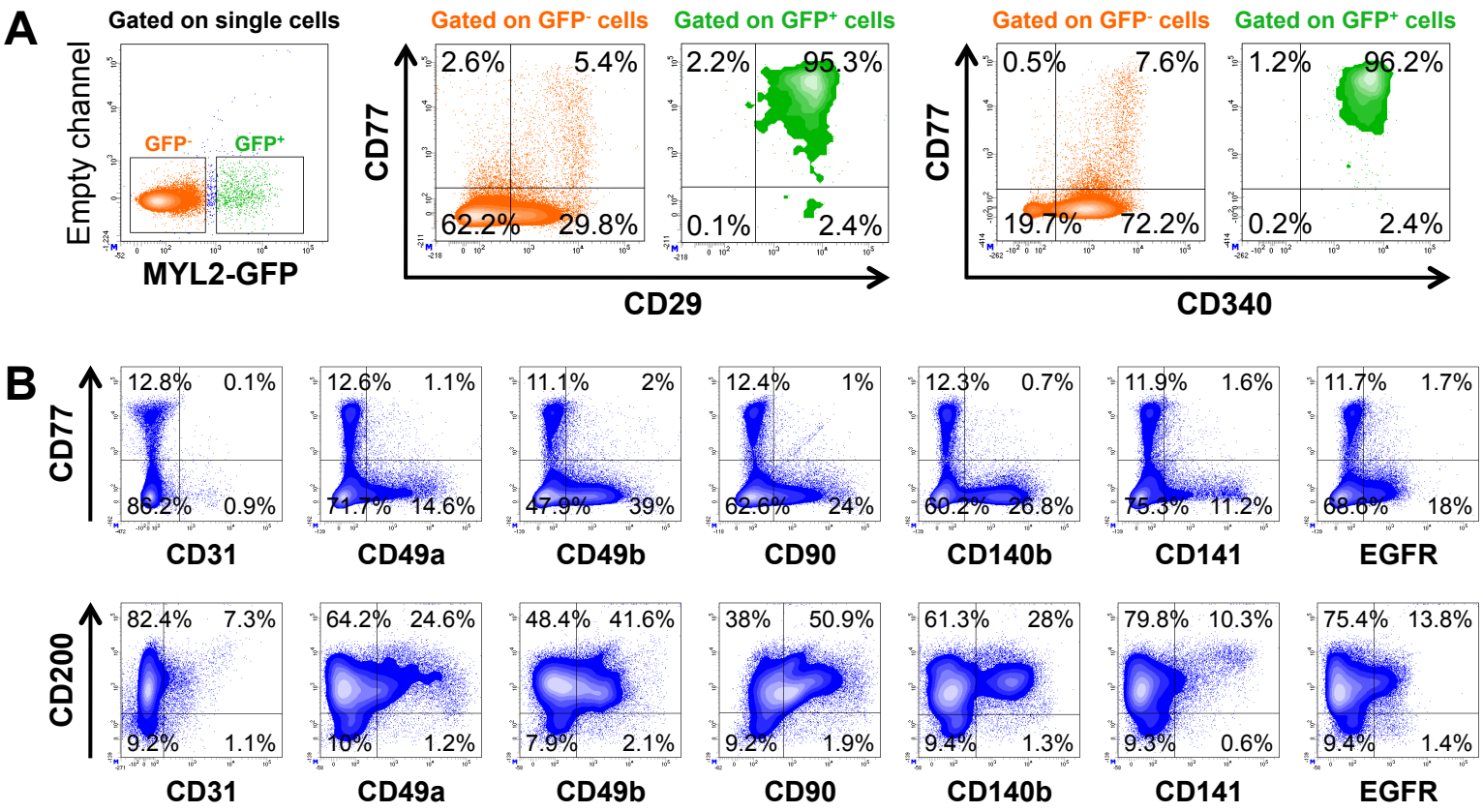
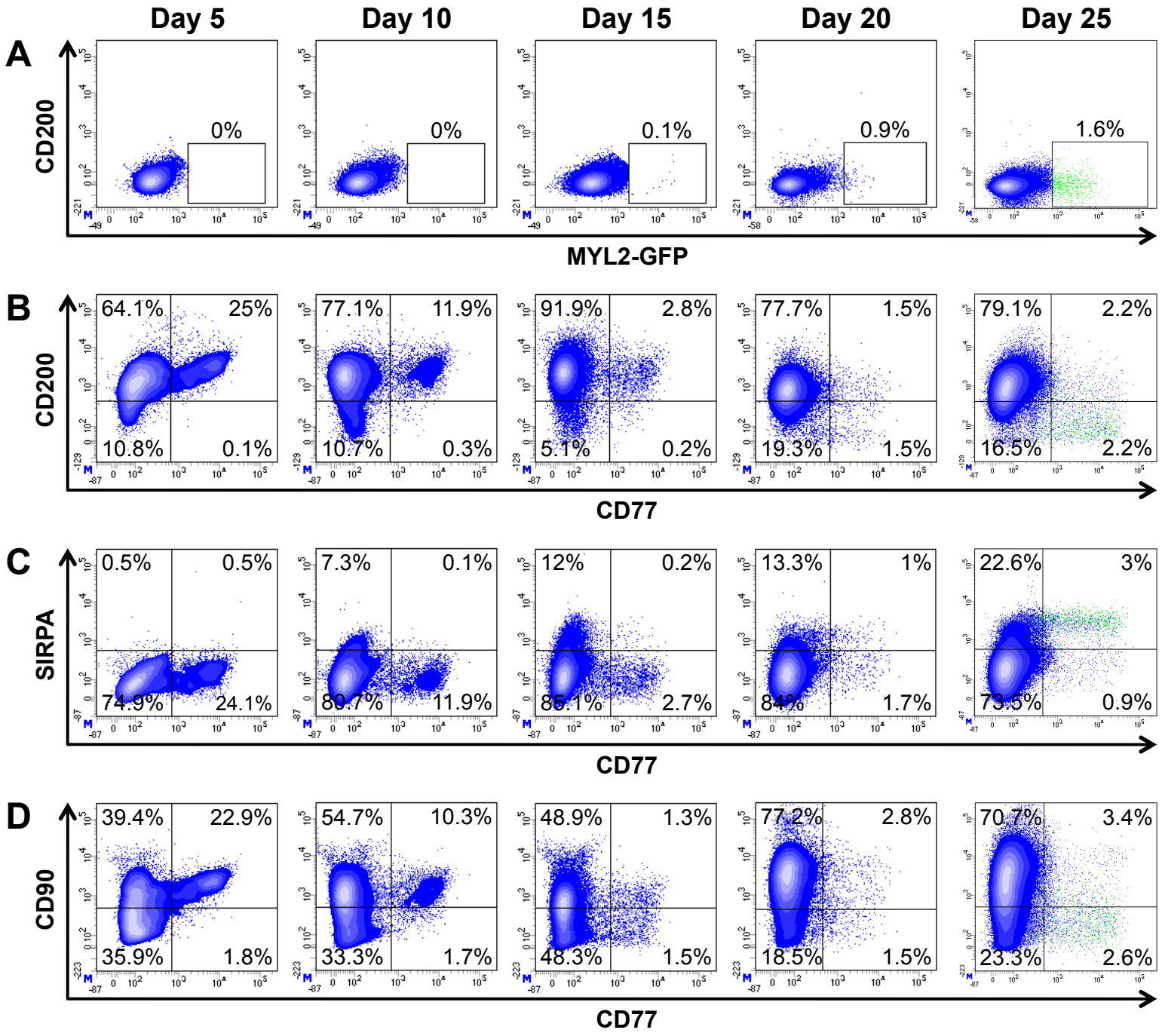

A
Gated on single cells
Empty channel
GFP-
GFP+
MYL2-GFP
Gated on GFP- cells
Gated on GFP+ cells
CD77
2.6%
5.4%
62.2%
29.8%
2.2%
95.3%
0.1%
2.4%
CD29
Gated on GFP- cells
Gated on GFP+ cells
CD77
0.5%
7.6%
19.7%
72.2%
1.2%
96.2%
0.2%
2.4%
CD340
B
CD77
12.8% 0.1% 86.2% 0.9%
12.6% 1.1% 71.7% 14.6%
11.1% 2% 47.9% 39%
12.4% 1% 62.6% 24%
12.3% 0.7% 60.2% 26.8%
11.9% 1.6% 75.3% 11.2%
11.7% 1.7% 68.6% 18%
CD31
CD49a
CD49b
CD90
CD140b
CD141
EGFR
CD200
82.4% 7.3% 9.2% 1.1%
64.2% 24.6% 10% 1.2%
48.4% 41.6% 7.9% 2.1%
38% 50.9% 9.2% 1.9%
61.3% 28% 9.4% 1.3%
79.8% 10.3% 9.3% 0.6%
75.4% 13.8% 9.4% 1.4%
CD31
CD49a
CD49b
CD90
CD140b
CD141
EGFR

Figure S5.



E Gated on VCAM1⁺ cells - Day 12

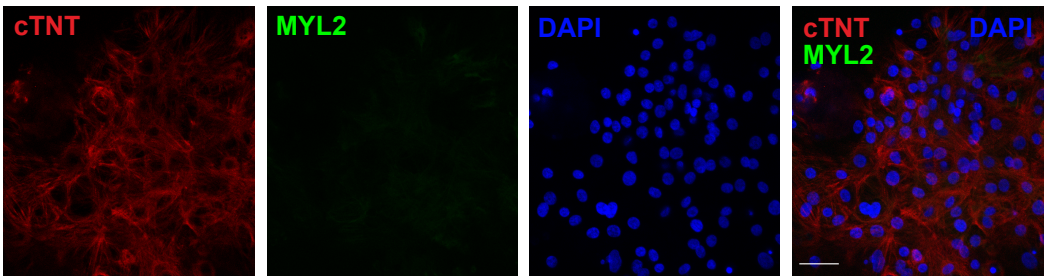
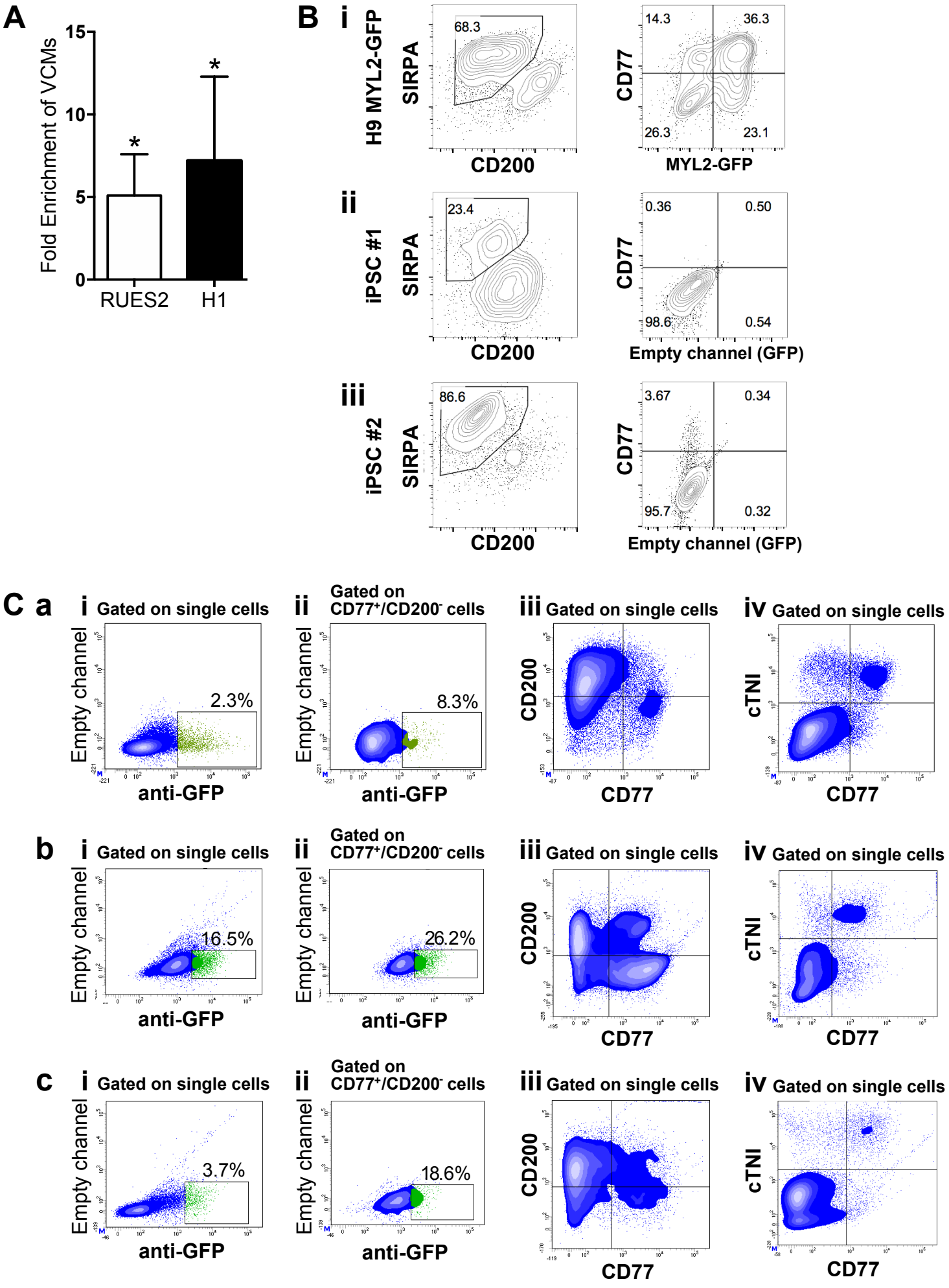


Figure S6.


A
Fold Enrichment of VCMs
15
10
5
0
*
*
RUES2
H1
B
i
H9 MYL2-GFP
SIRPA
68.3
CD200
CD77
14.3
36.3
26.3
23.1
MYL2-GFP
ii
iPSC #1
SIRPA
23.4
CD200
CD77
0.36
0.50
98.6
0.54
Empty channel (GFP)
iii
iPSC #2
SIRPA
86.6
CD200
CD77
3.67
0.34
95.7
0.32
Empty channel (GFP)
C
a
i Gated on single cells
Empty channel
2.3%
anti-GFP
ii Gated on CD77+/CD200- cells
Empty channel
8.3%
anti-GFP
iii Gated on single cells
CD200
CD77
iv Gated on single cells
cTNI
CD77
b
i Gated on single cells
Empty channel
16.5%
anti-GFP
ii Gated on CD77+/CD200- cells
Empty channel
26.2%
anti-GFP
iii Gated on single cells
CD200
CD77
iv Gated on single cells
cTNI
CD77
c
i Gated on single cells
Empty channel
3.7%
anti-GFP
ii Gated on CD77+/CD200- cells
Empty channel
18.6%
anti-GFP
iii Gated on single cells
CD200
CD77
iv Gated on single cells
cTNI
CD77

Supplemental Figure Legends

Figure S1. Verification of H9 MYL2-GFP BAC reporter line pluripotency and cardiac differentiation capacity. Related to Figure 1.
(A) Immunostaining for intracellular pluripotency markers NANOG (red), and OCT4 (green) in H9 MYL2-GFP BAC transgenic clone #3 hESCs. DNA stained with DAPI and merged images are shown. Scale bar, 25 μm. Images are representative of a minimum of three independent clones.
(B) Flow cytometric assessment of the cell surface pluripotency marker TRA-1-60 in H9 MYL2-GFP BAC transgenic clone #3 hESCs. The fibroblast marker CD13 was used as a negative control. Isotype controls were used to define the cytometric gates. Data are representative of a minimum of three independent clones.
(C) Giemsa-banding chromosomal preparations of H9 MYL2-GFP BAC transgenic clone #3 demonstrate a normal karyotype. All clones analyzed demonstrated a normal karyotype after transfection.
(D) A schematic representation of the embryoid body (EB) cardiac differentiation protocol (modified from [29]). In brief, cell clusters were washed and cultured in 6-well ultra-low attachment plates (Corning) in 2 mL differentiation media (DM) consisting of StemPro®-34 serum-free media (SFM) (Thermo Fisher Scientific), supplemented with 2 mM L-glutamine (Thermo Fisher Scientific), 50 μg/mL L-ascorbic acid (Sigma-Aldrich), 150 μg/mL transferrin (Roche), and $4x10^{-4}$ M monothioglycerol (MTG) (Sigma-Aldrich), and containing 10 ng/mL bone morphogenetic protein 4 (BMP-4) (R&D Systems). Following 24 hours incubation, on day 1, cell aggregates were induced with the addition of 1 mL DM containing a final concentration of 10 ng/mL BMP-4, 5 ng/mL basic fibroblast growth factor (bFGF), and 3 ng/mL Activin A (R&D Systems). On day 3 and day 5, EBs were harvested and resuspended in DM supplemented with 150 ng/mL Dickkopf-related protein 1 (DKK-1), and 10 ng/mL vascular endothelial growth factor (VEGF) (R&D Systems). On days 8, 12 and 16, EBs were harvested and cultured in DM containing 5 ng/mL bFGF, and 10 ng/mL VEGF. On and after day 20, DM without additional factors was replaced every 4-5 days for the duration of the experiment.
(E) RT-qPCR analysis of cell preparations of H9 MYL2-GFP BAC transgenic clone #3 over time course of EB cardiac differentiation. Data were normalized to corresponding *ACTB* expression, and is relative to hESC-derived EBs at day 0. Gene expression in human 15-week fetal atrial and ventricular tissue and human adult atrial and ventricular tissue was normalized to *GAPDH*. Error bars, mean ± SD (n=3 technical replicates). Data are representative of a minimum of three independent biological replicates.
(F) Flow cytometric quantification of MYL2-H2B-GFP (left column), cardiac troponin I (cTNI) (middle column), and MYL2-H2B-GFP/cTNI co-expression in three (samples 1-3) independent cardiac differentiation experiments of H9 MYL2-GFP hESCs at day 25 of differentiation.

Figure S2. Flow cytometry cell gating strategy. Related to Figure 1 and Figure 2.
(A) Parental H9 and MYL2-GFP cells at day 25 of cardiac differentiation were used to define the gating strategy used throughout all flow cytometry experiments. Top panel: (i-ii) All events were first analyzed by forward (FSC) and size (SSC) scatter properties to exclude doublets. (iii) After gating on singlets, unstained H9 cells were analyzed for background signal to further discriminate autofluorescent debris. Single cells were then analyzed in (iv) H9 and (v) MYL2 differentiated cells for GFP expression.
(B) For the sorting of $SIRPA^+/GFP^+$ and $SIRPA^+/GFP^-$ cells, single cells were first analyzed for the expression of SIRPA. (i) An isotype control was used to draw the gates for the detection of (ii) $SIRPA^{Bright}$ ($SIRPA^+$) cells. After gating of $SIRPA^+$ cells, (iii) GFP^+ and GFP^- cells were sorted.

Figure S3. Potential positive and negative cell-surface marker candidates to identify hESC-derived ventricular cardiomyocytes. Related to Figure 3.
Flow cytometric histogram plots of the highest ranked positive or negative cell-surface marker candidates within the MYL2-GFP^+ (GFP^+) (green) and MYL2-GFP^- (GFP^-) (orange) populations. Median fluorescence intensity (MFI) ratio and percent (%) frequency of each marker on each cell population stained are detailed in the table below each plot.

Figure S4. Multi-color validation by flow cytometric analysis of positive and negative cell-surface marker candidates to identify hESC-derived ventricular cardiomyocytes. Related to Figure 4.

Cells were analyzed at day 25 of cardiac differentiation. Data are representative of a minimum of three independent biological replicates.

(A) Left panel: MYL2-GFP$^-$ (GFP$^-$) and MYL2-GFP$^+$ (GFP$^+$) cell populations were defined within single cells. Contour plots depict expression of CD77 together with other positive marker candidates, CD29 (middle panel); or CD340 (right panel) within the GFP$^-$ (orange) and GFP$^+$ (green) cell populations.

(B) The expression of CD77 (top panels) and CD200 (bottom panels) was analyzed within single cells in relation to negative marker candidates: CD31, CD49a, CD49b, CD90, CD140b, CD141, and EGFR.

Figure S5. Temporal expression of cell-surface marker signature.

Data are representative of a minimum of three independent biological replicates.

(A) MYL2-GFP expression was analyzed within single cells over the time course of embryoid body cardiac differentiation (Day 5-25). Numbers indicate the percentage of GFP$^+$ cells.

(B-D) The differential expression of (B) CD200 and CD77; (C) SIRPA and CD77; and (D) CD90 and CD77 was analyzed within single cells over the time course of cardiac differentiation (Day 5-25). Green dots: overlay of MYL2-GFP$^+$ cells.

(E) Immunofluorescence analysis of H9 hESC-derived VCAM1$^+$-sorted cells after 12 days of cardiac differentiation. Cardiomyocytes were stained with cardiac troponin T (cTNT). No MYL2 expression was observed. DNA was stained with DAPI. Scale bar, 50 μm.

Figure S6. Enrichment of ventricular cardiomyocytes in human pluripotent stem cell lines and in monolayer cultures.

(A) Cells derived from RUES2 and H1 hESCs at day 25 of cardiac differentiation (for specific differentiation conditions, see Supplemental Experimental Procedures) were sorted for CD77$^+$/CD200$^-$ and analyzed for MYL2 expression. Following the sort, the percentage of cells expressing MYL2 was normalized to percentage of cells expressing MYL2 prior to sorting, and expressed as fold-enrichment for ventricular cardiomyocytes (VCMs). Error bars, mean ± SD. *$P \leq 0.05$ by paired *t*-test (n=3 independent biological replicates).

(B) Cells derived from **(i)** H9 MYL2-GFP hESCs, **(ii)** human induced pluripotent stem cell (iPSC) line 1 (#1), and **(iii)** human iPSC line 2 (#2) at day 25 of cardiac differentiation were first analyzed for the expression of SIRPA and absence of CD200 (left plot). After gating of SIRPA$^+$/CD200$^-$ cells, putative cardiomyocyte populations were subsequently analyzed for the expression of CD77 (right plot). Data are representative of a minimum of three independent biological replicates.

(C) Multicolor flow cytometry was used to simultaneously analyze the expression of (i-ii) MYL2-GFP (anti-GFP), (iii) surface markers CD77 and CD200, and (iv) CD77 and the intracellular cardiomyocyte marker, cardiac troponin I (cTNI) at day 60 of monolayer differentiation in three **(a, b,** and **c)** independent experiments.

Supplemental Tables

Table S1. (Excel File)
Heat map depicting the results from the cell-surface marker screen. Related to Figure 3.
Heat map detailing the expression of 242 cell-surface markers on MYL2-GFP^{+} (GFP^{+}) and MYL2-GFP^{-} (GFP^{-}) cell populations generated from the H9 MYL2-GFP reporter cell line after 25 days of cardiac differentiation. Markers are plotted by their median fluorescence intensity (MFI) ratio, and percent (%) frequency on each cell population stained with individual antibodies (BD Biosciences Lyoplate™ Human Cell Surface Marker Screening Panel). Isotype controls were used to normalize the MFI values and to draw the gates for proper discrimination between positive and negative cells.

Table S2. Primer sequences used for RT-qPCR analysis.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
ACTB	CTCACCATGGATGATGATATCGC	AGGAATCCTTCTGACCCATGC
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
GJA5	ATCACACCGGAAATCAGCCTG	CCGTGGTAGGCAAGGTCTG
ISL1	GACGGTGGCTTACAGGCTAA	CAGTGGAATTAGAGCCCGGT
MYH6	GCCCTTTGACATTCGCACTG	GGTTTCAGCAATGACCTTGCC
MYH7	CTTTGCTGTTATTGCAGCCATT	AGATGCCAACTTTCCTGTTGC
MYL2	TTGGGCGAGTGAACGTGAAAA	CCGAACGTAATCAGCCTTCAG
MYL7	TTCACCGTCTTCCTCACGCT	CATGGGTGTCAGGGCGAAC
NKX2-5	TATCCACGTGCCTACAGCGA	GTTGTCCGCCTCTGTCTTCT
NPPA	CAACGCAGACCTGATGGATTT	AGCCCCCGCTTCTTCATTC
T	AGACCCAGTTCATAGCGGTG	GGCACCTCCAAACTGAGGAT
TNNI3	CCTCACTGACCCTCCAAACG	GCATAAGCGCGGTAGTTGGA
TNNT2	GTGGGAAGAGGCAGACTGAG	ATAGATGCTCTGCCACAGC

Table S3. Antibodies used for flow cytometry.

Marker	Isotype	Clone	Fluorochrome	Manufacturer	Cat. No.
CD29	Mouse IgG_1	MAR4	PE	BD Biosciences	555443
CD31	Mouse IgG_1	WM59	BV605	BD Biosciences	562855
CD49a	Mouse IgG_1	SR84	PE	BD Biosciences	559596
CD49b	Mouse IgG_{2a}	12F1	PE	BD Biosciences	555669
CD77	Mouse IgM	5B5	BV510	BD Biosciences	563630
CD77	Mouse IgM	5B5	PE-CF594	BD Biosciences	563631

CD90	Mouse IgG_1	5E10	BV421	BD Biosciences	562556
CD140b	Mouse IgG_{2a}	28D4	PE	BD Biosciences	558821
CD141	Mouse IgG_1	1A4	PE	BD Biosciences	559781
CD172a/b (SIRPA)	Mouse IgG_1	SE5A5	PE	BD Biosciences	563441
CD200	Mouse IgG_1	MRC OX-104	PE-Cy7	BD Biosciences	562125
CD340	Mouse IgG_1	Neu 24.7	APC	BD Biosciences	340554
EGFR	Mouse IgG_{2b}	EGFR.1	PE	BD Biosciences	555997
cTNI	Mouse IgG_{2b}	C5	Alexa Fluor® 647	BD Biosciences	564409
VCAM1	Mouse IgG_1	STA	PE	BioLegend	305805
MYL2 **(Secondary)**	Rabbit IgG (Donkey IgG)	- -	- (Alexa Fluor® 488)	Proteintech (Thermo Fisher Scientific)	10906-1-AP (R37118)
Mouse IgG_1 Isotype Control	-	MOPC-21	PE	BD Biosciences	555749
Mouse IgG_1 Isotype Control	-	MOPC-21	PE-Cy7	BD Biosciences	557872

Supplemental Movie

Supplemental Movie 1.
Embryoid bodies derived from H9 MYL2-GFP hESCs. Related to Figure 1.
Epifluorescence analysis of embryoid bodies (EBs) derived from H9 MYL2-GFP hESCs, at day 25 of cardiac differentiation, confirmed nuclear GFP expression in cells throughout the EBs.

Supplemental Experimental Procedures

Pluripotent properties of H9 MYL2-GFP BAC transgenic clones

To verify that the transgenic hESC clones generated retained the unique properties of the parental H9 hESC line, undifferentiated hESCs were stained by immunocytochemistry for specific hESC markers NANOG (1:100 anti-NANOG Alexa Fluor® 647; BD Biosciences, 561300) and OCT4 (1:100 anti-OCT4 Alexa Fluor® 488; BD Biosciences, 561628), and flow cytometry for CD13 (anti-CD13 PerCP-Cy™5.5; BD Biosciences, 562626) and TRA-1-60 (anti-TRA-1-60 PE; BD Biosciences, 562626), as detailed in Experimental Procedures.

Maintenance and cardiac differentiation of human pluripotent stem cell (hPSC) lines

RUES2 and H1 control hESCs, and control iPSC lines were maintained on Matrigel™-coated tissue culture plates in irradiated mouse embryonic fibroblast (MEF) (GlobalStem)-conditioned hESC media consisting of DMEM/F-12 supplemented with 20% (v/v) KnockOut™ Serum Replacement, 100 μM non-essential amino acids, 2 mM L-glutamine, 55 μM β-mercaptoethanol (all from Thermo Fisher Scientific), and 20 ng/mL human basic fibroblast growth factor (bFGF) (PeproTech). Cells were passaged as small colonies, following dissociation with 1 mg/mL dispase (Thermo Fisher Scientific).

Prior to differentiation, hPSCs were cultured on MEFs in hESC media for 2-3 days to 90% confluence. Embryoid bodies (EBs) were differentiated to the cardiovascular lineage as described for hESCs in Experimental Procedures and Figure S1D. In brief, EBs were generated by dissecting hESC colonies into small clusters using an EZPassage™ Stem Cell Passaging Tool (Thermo Fisher Scientific), which were collected by gentle scraping. Cell clusters were washed and cultured in 6-well ultra-low attachment plates (Corning) in 2 mL differentiation media (DM) consisting of StemPro®-34 serum-free media (SFM) (Thermo Fisher Scientific), supplemented with 2 mM L-glutamine (Thermo Fisher Scientific), 50 μg/mL L-ascorbic acid (Sigma-Aldrich), 150 μg/mL transferrin (Roche), and $4x10^{-4}$ M monothioglycerol (MTG) (Sigma-Aldrich), and containing 10 ng/mL bone morphogenetic protein 4 (BMP-4) (R&D Systems). Following 24 hours incubation, on day 1, cell aggregates were induced with the addition of 1 mL DM containing a final concentration of 10 ng/mL BMP-4, 5 ng/mL bFGF, and 3 ng/mL Activin A (R&D Systems). On day 3 and day 5, EBs were harvested and resuspended in DM supplemented with 150 ng/mL Dickkopf-related protein 1 (DKK-1), and 10 ng/mL vascular endothelial growth factor (VEGF) (R&D Systems). On days 8, 12 and 16, EBs were harvested and cultured in DM containing 5 ng/mL bFGF, and 10 ng/mL VEGF. On and after day 20, DM without additional factors was replaced every 4-5 days for the duration of the experiment.

On day 25 of cardiac differentiation, EBs generated from hPSC differentiation experiments were harvested and dissociated to single cells. Briefly, EBs were washed and incubated in dissociation solution consisting of 1 mg/mL collagenase type II (Worthington) in Hank's balanced salt solution (Corning) (Formulation: 5.36 mM KCl; 0.44 mM KH_2PO_4; 136 mM NaCl; 0.39 mM Na_2HPO_4; 4.16 mM $NaHCO_3$; 5.55 mM D-glucose) with 5 mM HEPES (Corning), overnight at room temperature with gentle shaking. On the following day, an equal amount of dissociation solution supplemented with 1 mg/mL bovine serum albumin (Thermo Fisher Scientific), 0.1 mM EGTA (Sigma-Aldrich), and 10 mM taurine (Sigma-Aldrich) was added to the cell suspension, and EBs were pipetted gently to dissociate the cells. After dissociation, cells were centrifuged (200 × g for 5 minutes), filtered (100 μm cell strainer) and used for analysis.

Cardiac differentiation of hESCs by monolayer culture

H9 MYL2-GFP hESCs were maintained in Essential 8 (E8) media, split at 1:10 or 1:12 ratios using 0.5 M EDTA, and grown for 4 days (~85% confluence) prior to cardiac differentiation as previously described [14]. In brief, medium was changed to RPMI 1640 with L-Glutamine (Thermo Fisher Scientific), supplemented with 500 μg/mL recombinant human albumin (Sigma-Aldrich), and 213 μg/mL L-ascorbic acid (Sigma-Aldrich). On days 0-2, medium was supplemented with 6 μM CHIR99021 (Selleck). On day 3, medium was supplemented with 5 μM IWR-1 (Sigma-Aldrich). On and after day 5, differentiation media without additional factors was replaced every 48 hours for the duration of the experiment. At

indicated time points, cells were dissociated with TryPLE Express and used for multi-color flow cytometry. As the MYL2-GFP signal was weak following monolayer differentiation, an anti-GFP antibody was needed to detect MYL2-GFP^{+} cells.