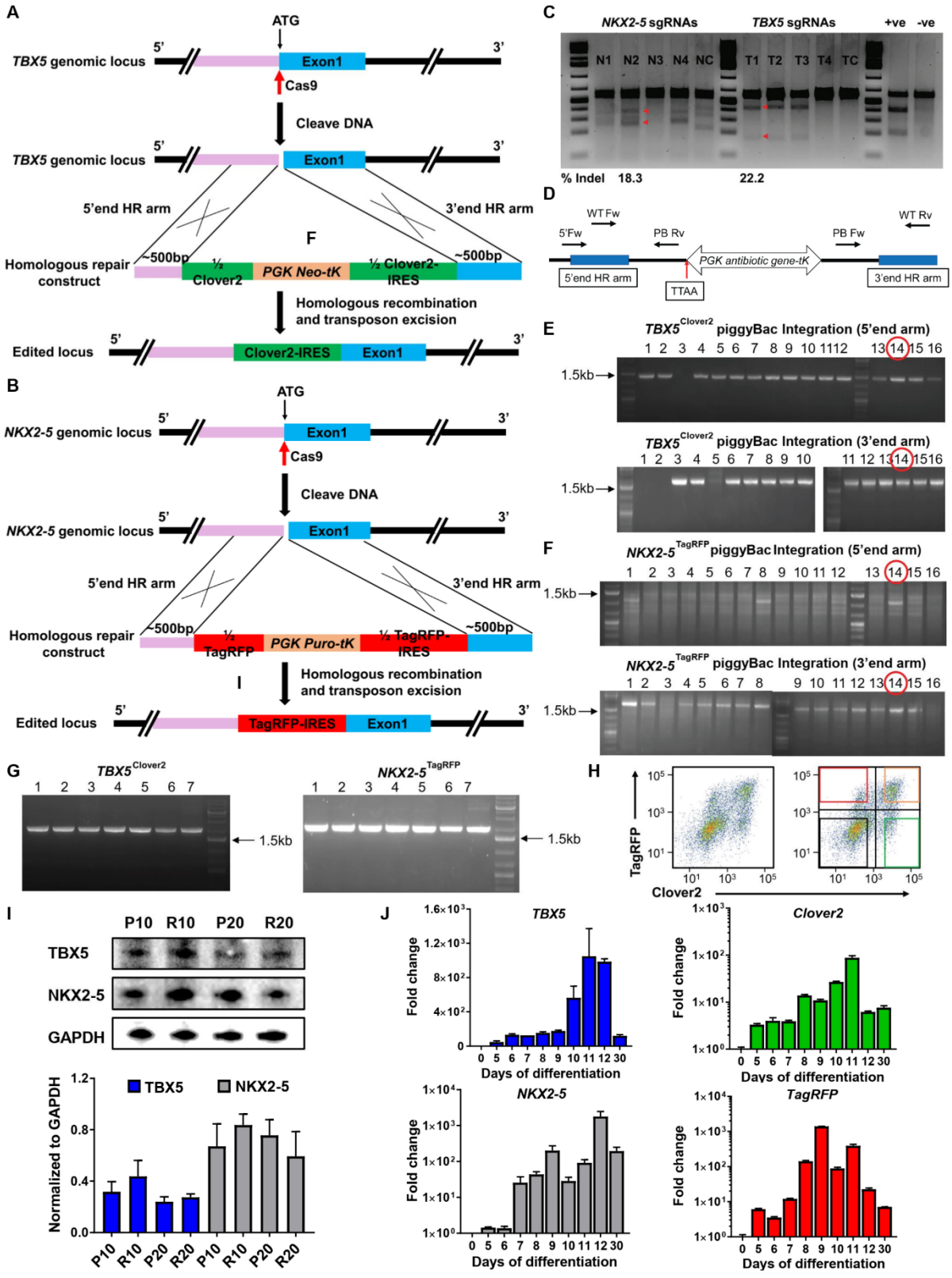
A
ATG
TBX5 genomic locus
5'
3'
Exon1
Cas9
Cleave DNA
5'end HR arm
3'end HR arm
Homologous repair construct
~500bp
½ Clover2
PGK Neo-tK
½ Clover2-IRES
Homologous recombination and transposon excision
Edited locus
Clover2-IRES
B
NKX2-5 genomic locus
½ TagRFP
PGK Puro-tK
½ TagRFP-IRES
TagRFP-IRES
C
NKX2-5 sgRNAs
TBX5 sgRNAs
+ve
-ve
N1 N2 N3 N4 NC T1 T2 T3 T4 TC
% Indel 18.3 22.2
D
WT Fw
WT Rv
5'Fw
PB Rv
PB Fw
PGK antibiotic gene-tK
TTAA
E
TBX5Clover2 piggyBac Integration (5'end arm)
TBX5Clover2 piggyBac Integration (3'end arm)
1.5kb
F
NKX2-5TagRFP piggyBac Integration (5'end arm)
NKX2-5TagRFP piggyBac Integration (3'end arm)
G
TBX5Clover2
NKX2-5TagRFP
H
TagRFP
Clover2
I
P10 R10 P20 R20
TBX5
NKX2-5
GAPDH
Normalized to GAPDH
J
TBX5
Clover2
NKX2-5
TagRFP
Fold change
Days of differentiation

Figure S1 (Related to Figure 1). Generation of TBX5^{Clover2}/NKX2-5^{TagRFP} hiPSC double reporter.

(A-B) Schematic of introducing Clover2 and TagRFP into **(A)** TBX5 and **(B)** NKX2-5 locus, respectively.

(C) Determination of the indel percentage by SURVEYOR assay in HEK 293 cells. Red arrowheads indicate expected fragments for each locus. Numbers at bottom indicate Cas9-mediated cleavage efficiency (percentage of indel). The sgRNAs N2 and T1 were used for this study. HR: homologous recombination.

(D) Schematic of primer design for determination of genome-edited clones. WT, wild type; PB, piggyBac; Fw, forward primer; Rv, reverse primer (See also **Table S1**).

(E-F) PCR-based screening of homology-directed piggyBac transposon integration at 5'end HR arm (upper panels) and 3'end HR arm (lower panels) into **(E)** TBX5 and **(F)** NKX2-5 locus. Clone 14 (red circle) was selected for excision of piggyBac transposon.

(G) PCR-based screening of transposon-excised clones using WT primers.

(H) Representative fluorescence-activated cell sorting (FACS) plot (left) shows four discrete clusters of cells derived from TBX5^{Clover2}/NKX2-5^{TagRFP} hiPSC reporter during cardiac differentiation. The same FACS plot on the right shows the gating strategy for cell sorting (colored boxes) and analysis (black lines).

(I) Protein expression of TBX5 and NKX2-5 on days 10 and 20 hiPSC-CMs derived from the reporter (R) and parental lines (P) (n=3).

(J) Kinetic gene expression of TBX5, Clover2, NKX2-5, and TagRFP from three independent differentiation.

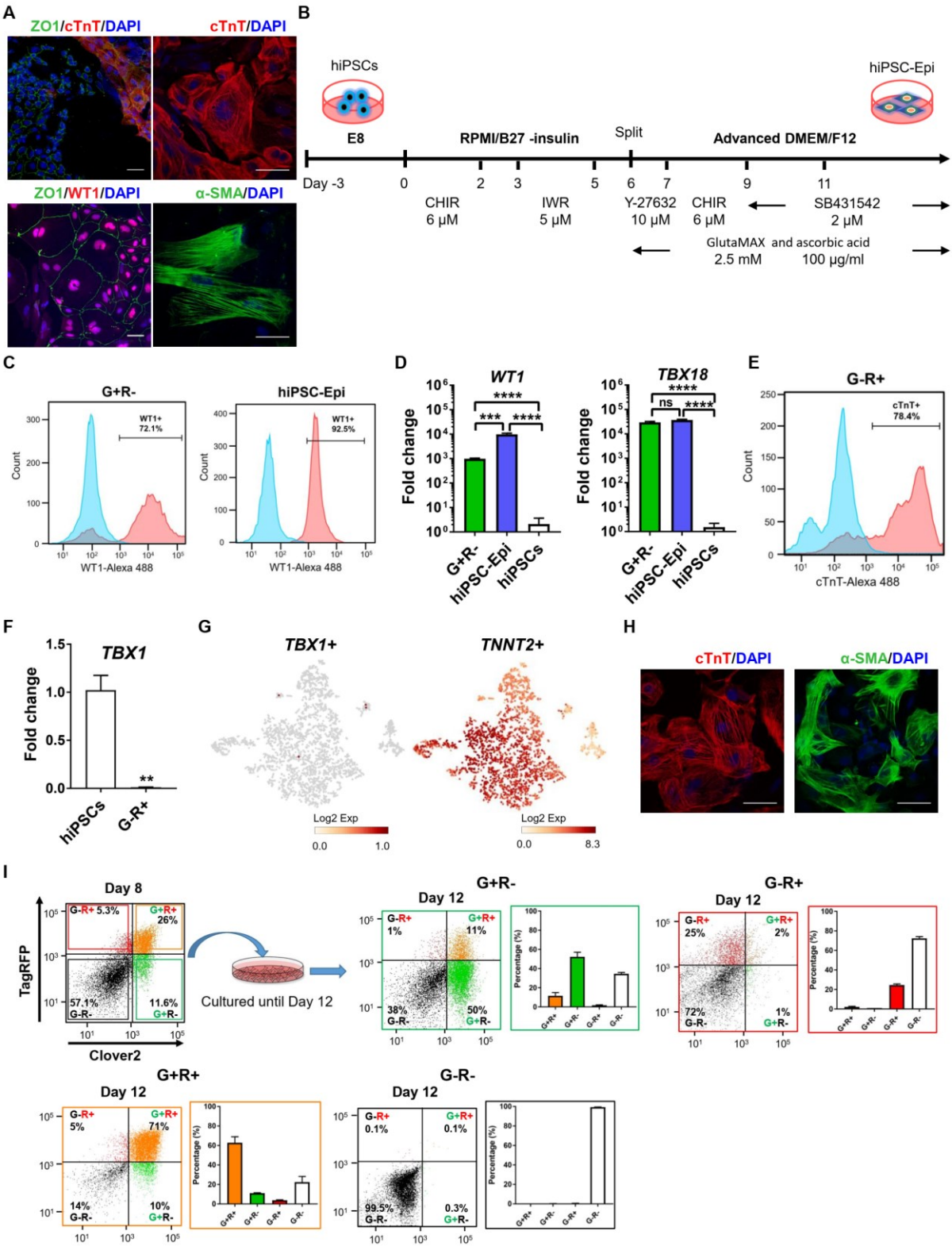
A
ZO1/cTnT/DAPI
cTnT/DAPI
ZO1/WT1/DAPI
α-SMA/DAPI
B
hiPSCs
hiPSC-Epi
E8
RPMI/B27 -insulin
Split
Advanced DMEM/F12
Day -3
0
2
3
5
6
7
9
11
CHIR 6 μM
IWR 5 μM
Y-27632 10 μM
CHIR 6 μM
SB431542 2 μM
GlutaMAX and ascorbic acid
2.5 mM
100 μg/ml
C
G+R-
hiPSC-Epi
WT1+ 72.1%
WT1+ 92.5%
Count
WT1-Alexa 488
D
WT1
TBX18
Fold change
G+R-
hiPSC-Epi
hiPSCs
E
G-R+
cTnT+ 78.4%
cTnT-Alexa 488
F
TBX1
Fold change
hiPSCs
G-R+
G
TBX1+
TNNT2+
Log2 Exp
H
cTnT/DAPI
α-SMA/DAPI
I
Day 8
TagRFP
Clover2
G-R+ 5.3%
G+R+ 26%
57.1% G-R-
11.6% G+R-
Cultured until Day 12
G+R-
Day 12
G-R+ 1%
G+R+ 11%
38% G-R-
50% G+R-
G-R+
Day 12
G-R+ 25%
G+R+ 2%
72% G-R-
1% G+R-
G+R+
Day 12
G-R+ 5%
G+R+ 71%
14% G-R-
10% G+R-
G-R-
Day 12
G-R+ 0.1%
G+R+ 0.1%
99.5% G-R-
0.3% G+R-
Percentage (%)

Figure S2 (Related to Figure 2). Determination of the identity of G+R- and G-R+ subpopulations.

(A) Immunostaining of day 30 G+R- cells showing a mixture of ZO1+ (green) and cardiac troponin T+ (cTnT, red) cells. ZO1+ cells are also WT1+ (red). Scale bars, 50 μm.

(B) Schematic of epicardial cell differentiation protocol.

(C) Flow cytometry of WT1 in day 30 G+R- and hiPSC-epicardial (hiPSC-Epi) cells.

(D) Expression of WT1 and TBX18 in day 30 G+R- subpopulation, hiPSC-Epi, and hiPSCs (n=3; ***$p<0.001$, ****$p<0.0001$, ns = not significant).

(E) Flow cytometry of cTnT in day 30 G-R+ subpopulation.

(F) Expression of TBX1 in hiPSCs and day 30 G-R+ subpopulation (n=3; **$p<0.01$).

(G) The t-distributed stochastic neighbor embedding (t-SNE) plot of day 30 cardiomyocytes showing TBX1+ cells (left) and TNNT2+ cells (right). Single cell RNA-seq dataset is from (Churko et al., 2018).

(H) Immunostaining of G-R+ cells showing differentiated cardiomyocytes (cTnT) and smooth muscle cells (α-SMA). Scale bars, 50 μm.

(I) Flow cytometry shows dynamic changes in the expression of TBX5 and NKX2-5 in each subpopulation from days 8 to 12 from three independent experiments.

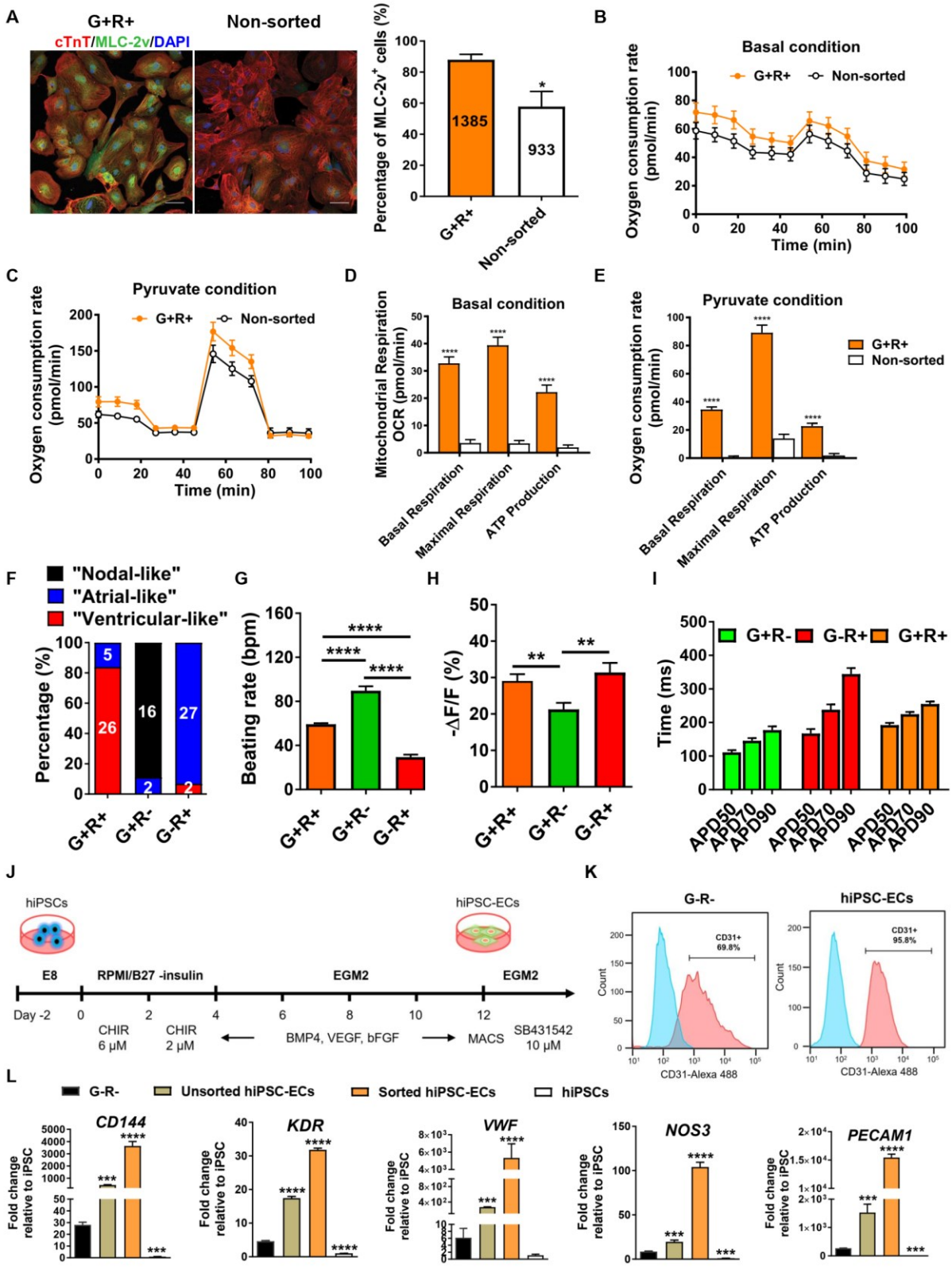
A
G+R+
Non-sorted
cTnT/MLC-2v/DAPI
Percentage of MLC-2v+ cells (%)
1385
933
G+R+
Non-sorted
B
Basal condition
G+R+
Non-sorted
Oxygen consumption rate (pmol/min)
Time (min)
C
Pyruvate condition
G+R+
Non-sorted
Oxygen consumption rate (pmol/min)
Time (min)
D
Basal condition
Mitochondrial Respiration OCR (pmol/min)
Basal Respiration
Maximal Respiration
ATP Production
E
Pyruvate condition
G+R+
Non-sorted
Oxygen consumption rate (pmol/min)
Basal Respiration
Maximal Respiration
ATP Production
F
"Nodal-like"
"Atrial-like"
"Ventricular-like"
Percentage (%)
5
26
16
2
27
2
G+R+
G+R-
G-R+
G
Beating rate (bpm)
G+R+
G+R-
G-R+
H
-ΔF/F (%)
G+R+
G+R-
G-R+
I
G+R-
G-R+
G+R+
Time (ms)
APD50
APD70
APD90
J
hiPSCs
hiPSC-ECs
E8
RPMI/B27 -insulin
EGM2
EGM2
Day -2
0
2
4
6
8
10
12
CHIR 6 μM
CHIR 2 μM
BMP4, VEGF, bFGF
MACS
SB431542 10 μM
K
G-R-
hiPSC-ECs
CD31+ 69.8%
CD31+ 95.8%
Count
CD31-Alexa 488
L
G-R-
Unsorted hiPSC-ECs
Sorted hiPSC-ECs
hiPSCs
CD144
KDR
VWF
NOS3
PECAM1
Fold change relative to iPSC

Figure S3 (Related to Figure 2). Characterization of G+R+ and G-R- subpopulations.

(A) Co-immunostaining of day 30 G+R+ and non-sorted cardiomyocytes for cardiac troponin T (cTnT, red) and ventricular myosin light chain 2 (MLC-2v, green). Scale bars, 50 μm. Average percentage of MLC-2v+ cells out of total cTnT+ cells. 1,385 cells from the G+R+ subpopulation and 933 cells from the non-sorted population were analyzed in 7 regions of interest from two independent experiments (*p<0.05).

(B-C) Representative Seahorse extracellular-flux assay traces measuring oxygen consumption rate under **(B)** basal and **(C)** pyruvate condition.

(D-E) Quantitative analysis of oxygen consumption rate under **(D)** basal and **(E)** pyruvate condition (n=8; ****p<0.0001).

(F) Percentage of subtypes in day 60 G+R+, G+R-, and G-R+ cardiomyocytes. Numbers indicate the recorded cell number.

(G-I) Quantification of beating rate, AP amplitude, and AP duration (APD) in day 60 G+R-, G-R+, and G+R+ cardiomyocytes (G+R-, n=18; G-R+, n=29; G+R+, n=31; **p<0.01, ****p<0.0001).

(J) Schematic of EC differentiation protocol.

(K) Flow cytometry analysis of CD31 expression in day 30 hiPSC-ECs and G-R- cells.

(L) Gene expression analysis of EC markers in day 30 G-R-, unsorted hiPSC-ECs, sorted hiPSC-ECs, and hiPSCs (n=3; ***p<0.001, ****p<0.0001, compared with G-R- group).

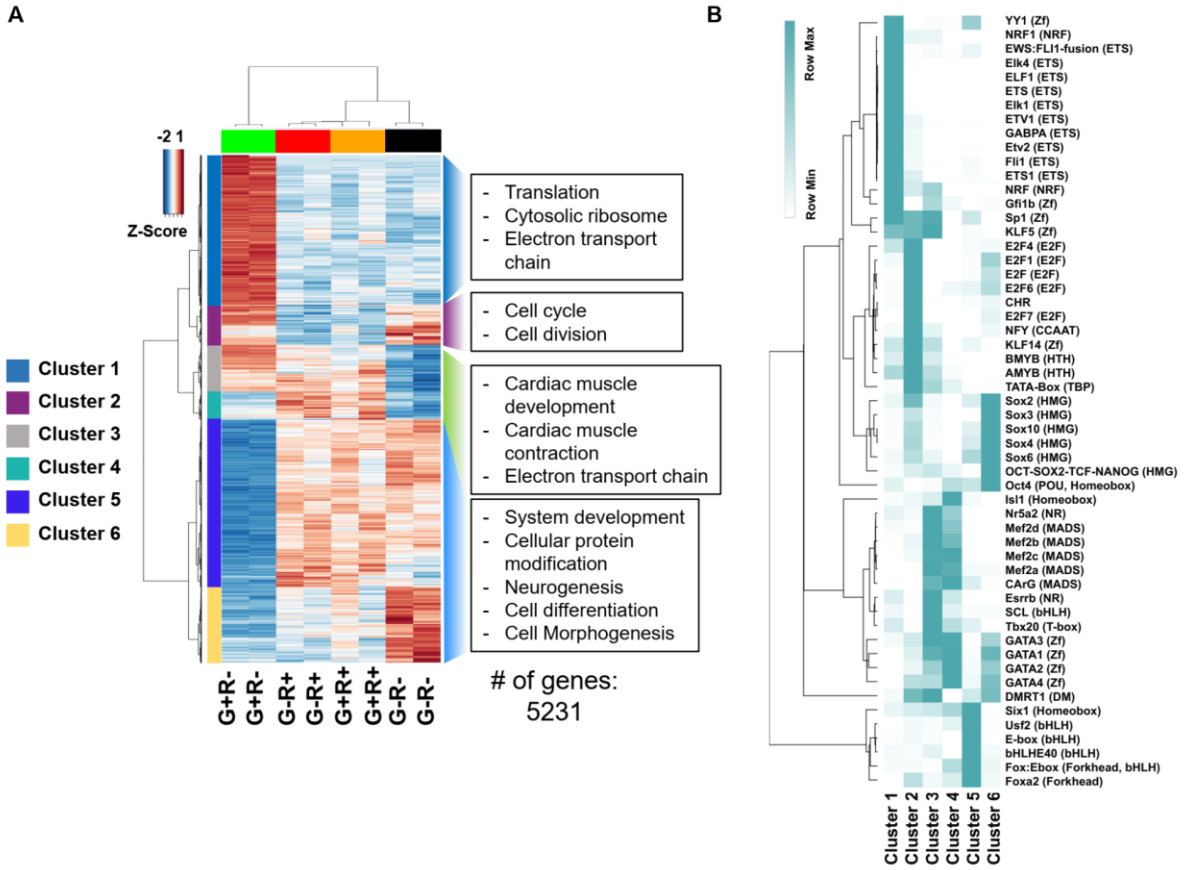
A
-2 1
Z-Score
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
G+R-
G+R-
G-R+
G-R+
G+R+
G+R+
G-R-
G-R-
- Translation
- Cytosolic ribosome
- Electron transport chain
- Cell cycle
- Cell division
- Cardiac muscle development
- Cardiac muscle contraction
- Electron transport chain
- System development
- Cellular protein modification
- Neurogenesis
- Cell differentiation
- Cell Morphogenesis
# of genes:
5231
B
Row Max
Row Min
YY1 (Zf)
NRF1 (NRF)
EWS:FLI1-fusion (ETS)
Elk4 (ETS)
ELF1 (ETS)
ETS (ETS)
Elk1 (ETS)
ETV1 (ETS)
GABPA (ETS)
Etv2 (ETS)
Fli1 (ETS)
ETS1 (ETS)
NRF (NRF)
Gfi1b (Zf)
Sp1 (Zf)
KLF5 (Zf)
E2F4 (E2F)
E2F1 (E2F)
E2F (E2F)
E2F6 (E2F)
CHR
E2F7 (E2F)
NFY (CCAAT)
KLF14 (Zf)
BMYB (HTH)
AMYB (HTH)
TATA-Box (TBP)
Sox2 (HMG)
Sox3 (HMG)
Sox10 (HMG)
Sox4 (HMG)
Sox6 (HMG)
OCT-SOX2-TCF-NANOG (HMG)
Oct4 (POU, Homeobox)
Isl1 (Homeobox)
Nr5a2 (NR)
Mef2d (MADS)
Mef2b (MADS)
Mef2c (MADS)
Mef2a (MADS)
CArG (MADS)
Esrrb (NR)
SCL (bHLH)
Tbx20 (T-box)
GATA3 (Zf)
GATA1 (Zf)
GATA2 (Zf)
GATA4 (Zf)
DMRT1 (DM)
Six1 (Homeobox)
Usf2 (bHLH)
E-box (bHLH)
bHLHE40 (bHLH)
Fox:Ebox (Forkhead, bHLH)
Foxa2 (Forkhead)
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6

Figure S4 (Related to Figure 3). Isolated subpopulations have differential transcriptome.

(A) Hierarchical clustering shows 6 different clusters and associated GO pathways.

(B) Heatmap shows prediction of transcription factor binding sites on the promotors of clustered genes.

Table S1 (Related to Figure 1). Design of primers for confirming gene integration and excision of transposon.

Primers	**Sequences**	**Notes**
TBX5 5'end HR arm forward	TTTTGTCCTCGCCAATCCTG	Testing TBX5 5'end HR arm integration
PiggyBac reverse	AGACCGATAAAACACATGCGTCAA	
PiggyBac forward	GTCCTAAATGCACAGCGACG	Testing TBX5 3'end HR arm integration
TBX5 WT reverse	TGTTAGCTGACCCCAAACGG	
TBX5 WT forward	AGGTGCTGGTAGCTGGAAAC	Testing piggyBac excision at TBX5 locus
TBX5 WT reverse	TGTTAGCTGACCCCAAACGG	
NKX2-5 5'end HR arm forward	CTCCAAACAGGGGAAACAAGGT	Testing NKX2-5 5'end HR arm integration
PiggyBac reverse	AGACCGATAAAACACATGCGTCAA	
PiggyBac forward	GTCCTAAATGCACAGCGACG	Testing NKX2-5 3'end HR arm integration
NKX2-5 WT reverse	TCTCAACTTCCTACCAGACCCA	
NKX2-5 WT forward	ACTGCTCATCGCTCCTGTCA	Testing piggyBac excision at NKX2-5 locus
NKX2-5 WT reverse	TCTCAACTTCCTACCAGACCCA	
HR, homologous recombination; WT, wild type		

Table S2 (Related to Figure 1). Statistical analysis of gene expression of subpopulations isolated on day 10. *P*-values are reported for one-way ANOVA with post-hoc Tukey test for multiple comparisons of gene expression between different subpopulations.

Gene	Population 1	Population 2	*P*-value	Gene	Population 1	Population 2	*P*-value
TBX5	G+R+	G+R-	0.0155	***MEF2C***	G+R+	G+R-	0.996
		G-R+	< 0.0001			G-R+	0.0153
		G-R-	< 0.0001			G-R-	0.5997
	G+R-	G-R+	< 0.0001		G+R-	G-R+	0.0205
		G-R-	< 0.0001			G-R-	0.4819
	G-R+	G-R-	0.9877		G-R+	G-R-	0.0031
NKX2-5	G+R+	G+R-	< 0.0001	***WT1***	G+R+	G+R-	< 0.0001
		G-R+	< 0.0001			G-R+	0.3655
		G-R-	< 0.0001			G-R-	> 0.9999
	G+R-	G-R+	< 0.0001		G+R-	G-R+	< 0.0001
		G-R-	0.5806			G-R-	< 0.0001
	G-R+	G-R-	< 0.0001		G-R+	G-R-	0.3648
GATA4	G+R+	G+R-	0.0488	***TBX18***	G+R+	G+R-	0.0002
		G-R+	0.1681			G-R+	0.6925
		G-R-	0.0009			G-R-	0.6212
	G+R-	G-R+	0.0024		G+R-	G-R+	< 0.0001
		G-R-	< 0.0001			G-R-	< 0.0001
	G-R+	G-R-	0.0135		G-R+	G-R-	0.9992
TNNT2	G+R+	G+R-	0.4786	***PECAM***	G+R+	G+R-	0.0164
		G-R+	0.0098			G-R+	0.9968
		G-R-	< 0.0001			G-R-	0.0137
	G+R-	G-R+	0.077		G+R-	G-R+	0.0215
		G-R-	< 0.0001			G-R-	0.9991
	G-R+	G-R-	< 0.0001		G-R+	G-R-	0.018
HCN4	G+R+	G+R-	0.679	***KDR***	G+R+	G+R-	0.0073
		G-R+	0.0488			G-R+	0.9891
		G-R-	0.0061			G-R-	0.0003
	G+R-	G-R+	0.0104		G+R-	G-R+	0.0108
		G-R-	0.0016			G-R-	0.0824
	G-R+	G-R-	0.453		G-R+	G-R-	0.0004
ISL1	G+R+	G+R-	0.0039				
		G-R+	0.0023				
		G-R-	0.0109				
	G+R-	G-R+	0.966				
		G-R-	0.8424				
	G-R+	G-R-	0.5981				

Table S4 (Related to STAR Methods). The oligonucleotides used for this study.

Gene	Source	Identifier
18s	ThermoFisher Scientific	Hs99999901_s1
TBX5	ThermoFisher Scientific	Hs00361155_m1
NKX2-5	ThermoFisher Scientific	Hs00231763_m1
GATA4	ThermoFisher Scientific	Hs00171403_m1
TNNT2	ThermoFisher Scientific	Hs00943911_m1
MEF2C	ThermoFisher Scientific	Hs00231149_m1
HCN4	ThermoFisher Scientific	Hs00975492_m1
ISL1	ThermoFisher Scientific	Hs00158126_m1
WT1	ThermoFisher Scientific	Hs01103751_m1
TBX18	ThermoFisher Scientific	Hs01385457_m1
PECAM1	ThermoFisher Scientific	Hs01065279_m1
KDR	ThermoFisher Scientific	Hs00911700_m1
KCNJ2	ThermoFisher Scientific	Hs01876357_s1
KCNJ3	ThermoFisher Scientific	Hs04334861_s1
KCNJ12	ThermoFisher Scientific	Hs00266926_s1
KCNJ4	ThermoFisher Scientific	Hs00705379_s1
MYL7	ThermoFisher Scientific	Hs01085598_g1
NR2F2	ThermoFisher Scientific	Hs00819630_m1
PITX2	ThermoFisher Scientific	Hs04234069_mH
HAND1	ThermoFisher Scientific	Hs02330376_s1
HAND2	ThermoFisher Scientific	Hs00232769_m1
KCNH2	ThermoFisher Scientific	Hs04234270_g1
KCNIP2	ThermoFisher Scientific	Hs01552688_g1
SCN5A	ThermoFisher Scientific	Hs00165693_m1
TCF21	ThermoFisher Scientific	Hs00162646_m1
TBX1	ThermoFisher Scientific	Hs00962558_g1
MYH11	ThermoFisher Scientific	Hs00975796_m1
SHOX2	ThermoFisher Scientific	Hs00243203_m1
ACTA2	ThermoFisher Scientific	Hs00426835_g1
IRX4	ThermoFisher Scientific	Hs00212560_m1
VWF	ThermoFisher Scientific	Hs01109446_m1
NOS3	ThermoFisher Scientific	Hs01574665_m1
CD144	ThermoFisher Scientific	Hs00901465_m1
MYL2	ThermoFisher Scientific	Hs00166405_m1
HCN1	ThermoFisher Scientific	Hs01085412_m1
TBX2	ThermoFisher Scientific	Hs00911929_m1
TBX3	ThermoFisher Scientific	Hs00195612_m1
CORIN	ThermoFisher Scientific	Hs00198141_m1
Clover2	ThermoFisher Scientific	Custom design
TagRFP	ThermoFisher Scientific	Custom design