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Supplemental Information

Effects of Cryopreservation on Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes for Assessing Drug Safety Response Profiles

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Figure S1

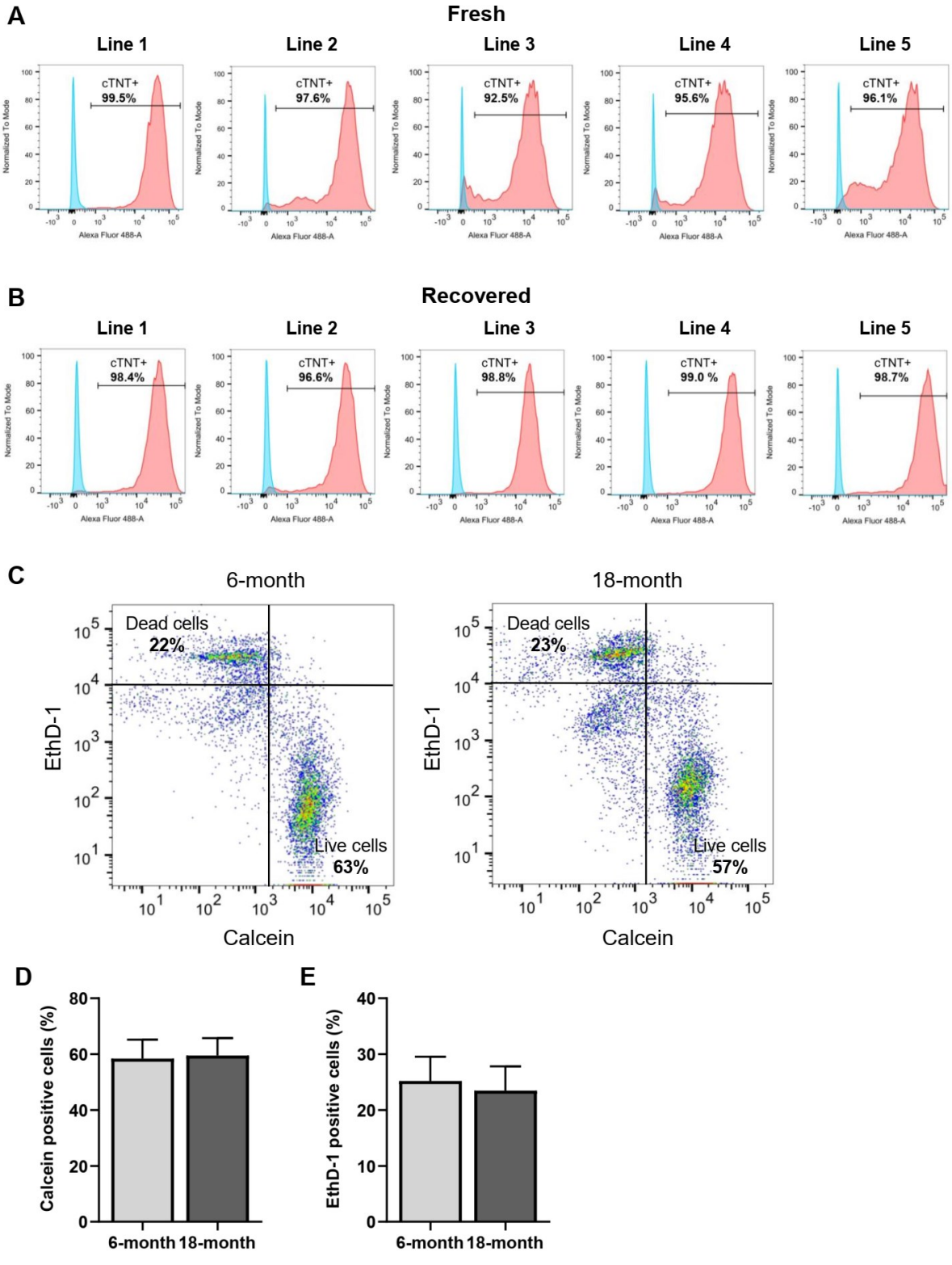
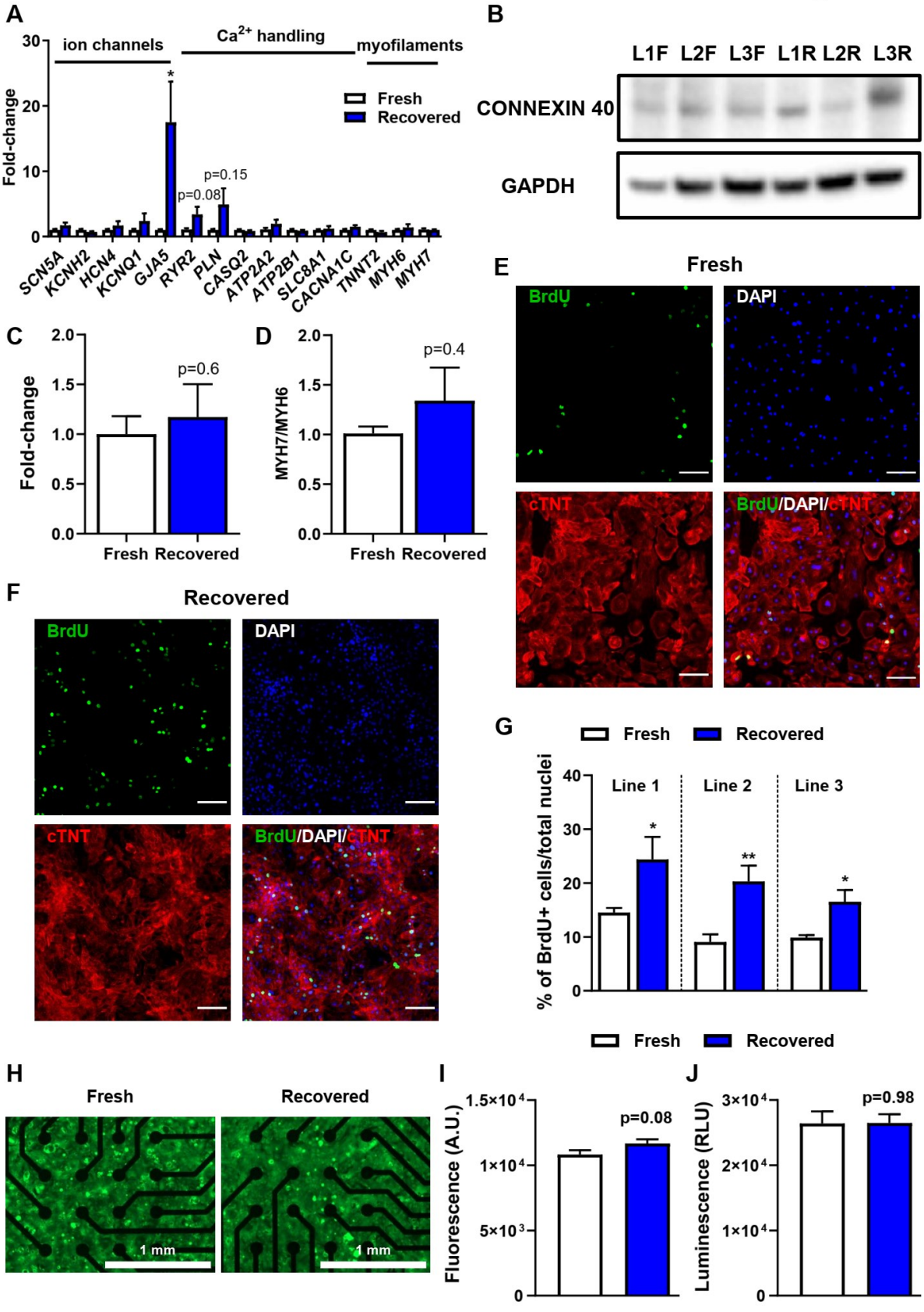
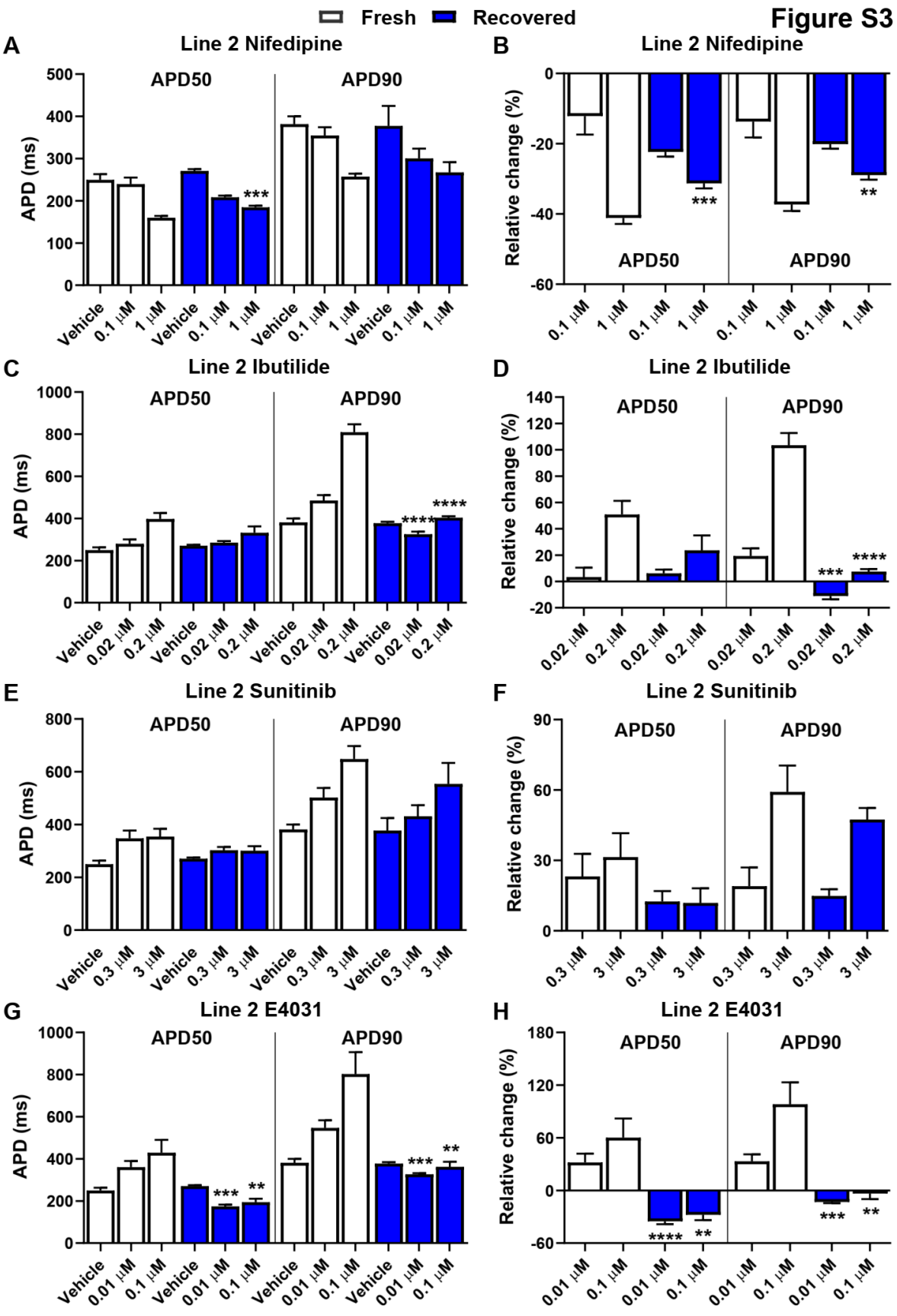

A
Fresh
Line 1
cTNT+ 99.5%
Line 2
cTNT+ 97.6%
Line 3
cTNT+ 92.5%
Line 4
cTNT+ 95.6%
Line 5
cTNT+ 96.1%
Normalized To Mode
Alexa Fluor 488-A
B
Recovered
Line 1
cTNT+ 98.4%
Line 2
cTNT+ 96.6%
Line 3
cTNT+ 98.8%
Line 4
cTNT+ 99.0 %
Line 5
cTNT+ 98.7%
C
6-month
Dead cells 22%
Live cells 63%
18-month
Dead cells 23%
Live cells 57%
EthD-1
Calcein
D
Calcein positive cells (%)
6-month
18-month
E
EthD-1 positive cells (%)
6-month
18-month

Figure S2


A
ion channels
Ca2+ handling
myofilaments
Fold-change
Fresh
Recovered
p=0.15
p=0.08
SCN5A
KCNH2
HCN4
KCNQ1
GJA5
RYR2
PLN
CASQ2
ATP2A2
ATP2B1
SLC8A1
CACNA1C
TNNT2
MYH6
MYH7
B
L1F L2F L3F L1R L2R L3R
CONNEXIN 40
GAPDH
C
Fold-change
p=0.6
Fresh Recovered
D
MYH7/MYH6
p=0.4
Fresh Recovered
E
Fresh
BrdU
DAPI
cTNT
BrdU/DAPI/cTNT
F
Recovered
BrdU
DAPI
cTNT
BrdU/DAPI/cTNT
G
Fresh
Recovered
% of BrdU+ cells/total nuclei
Line 1
Line 2
Line 3
H
Fresh
Recovered
1 mm
I
Fluorescence (A.U.)
p=0.08
J
Luminescence (RLU)
p=0.98


Fresh
Recovered
Figure S3
A
Line 2 Nifedipine
APD50
APD90
APD (ms)
Vehicle
0.1 μM
1 μM
B
Line 2 Nifedipine
Relative change (%)
C
Line 2 Ibutilide
0.02 μM
0.2 μM
D
Line 2 Ibutilide
E
Line 2 Sunitinib
0.3 μM
3 μM
F
Line 2 Sunitinib
G
Line 2 E4031
0.01 μM
H
Line 2 E4031

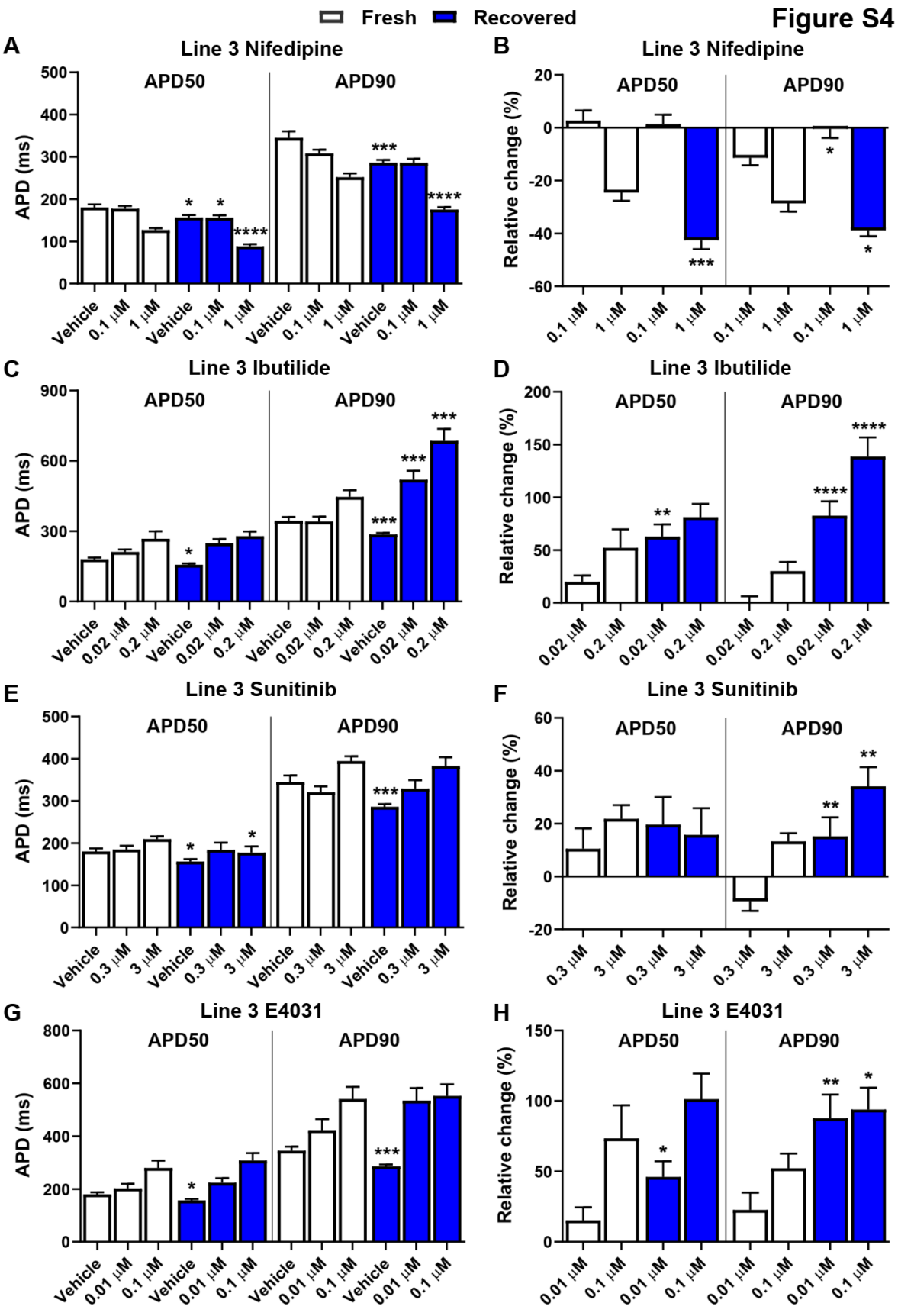

Fresh
Recovered
Figure S4
A
Line 3 Nifedipine
APD50
APD90
APD (ms)
Vehicle
0.1 μM
1 μM
B
Line 3 Nifedipine
Relative change (%)
C
Line 3 Ibutilide
0.02 μM
0.2 μM
D
Line 3 Ibutilide
E
Line 3 Sunitinib
0.3 μM
3 μM
F
Line 3 Sunitinib
G
Line 3 E4031
0.01 μM
0.1 μM
H
Line 3 E4031

Figure S5

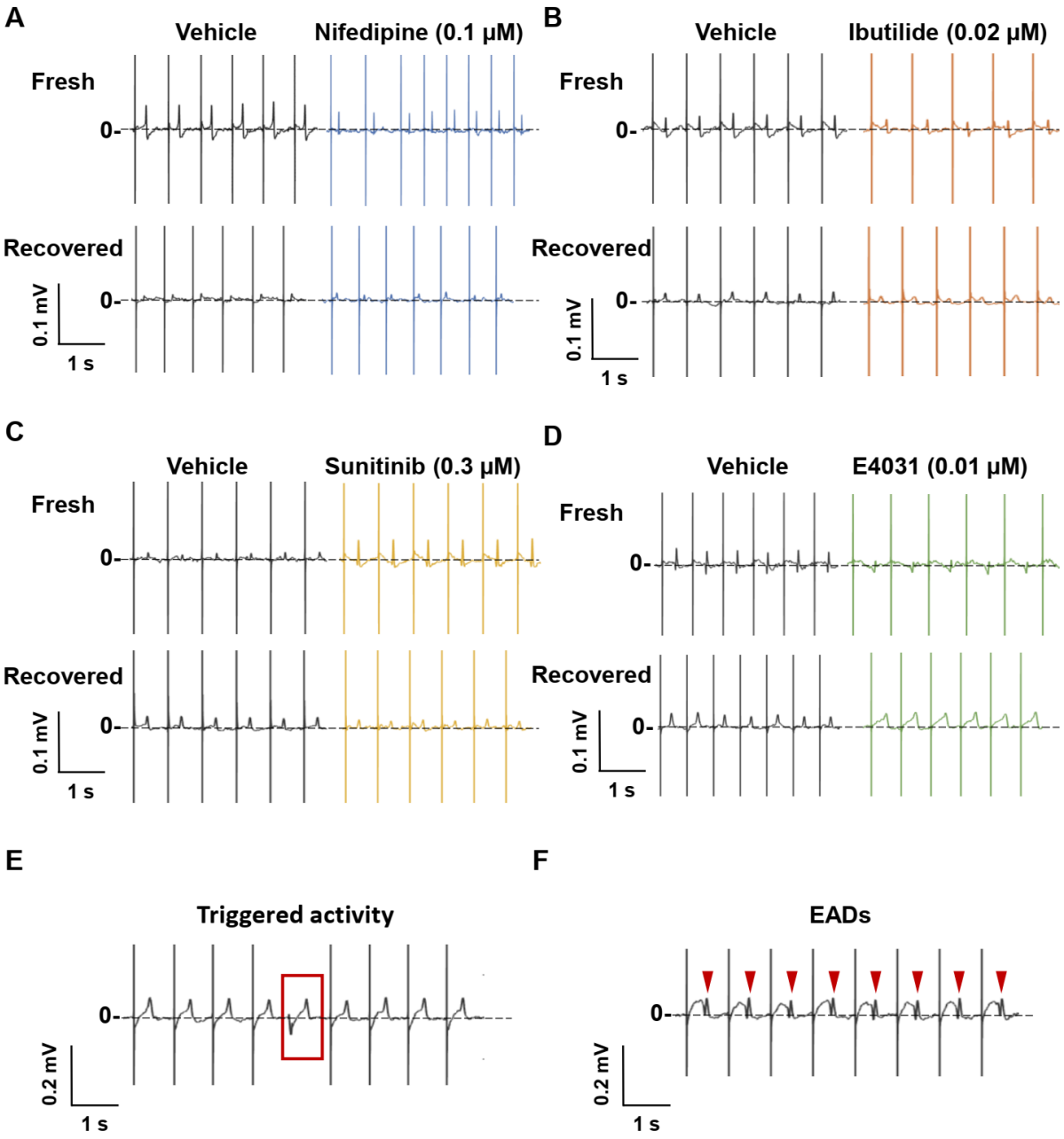
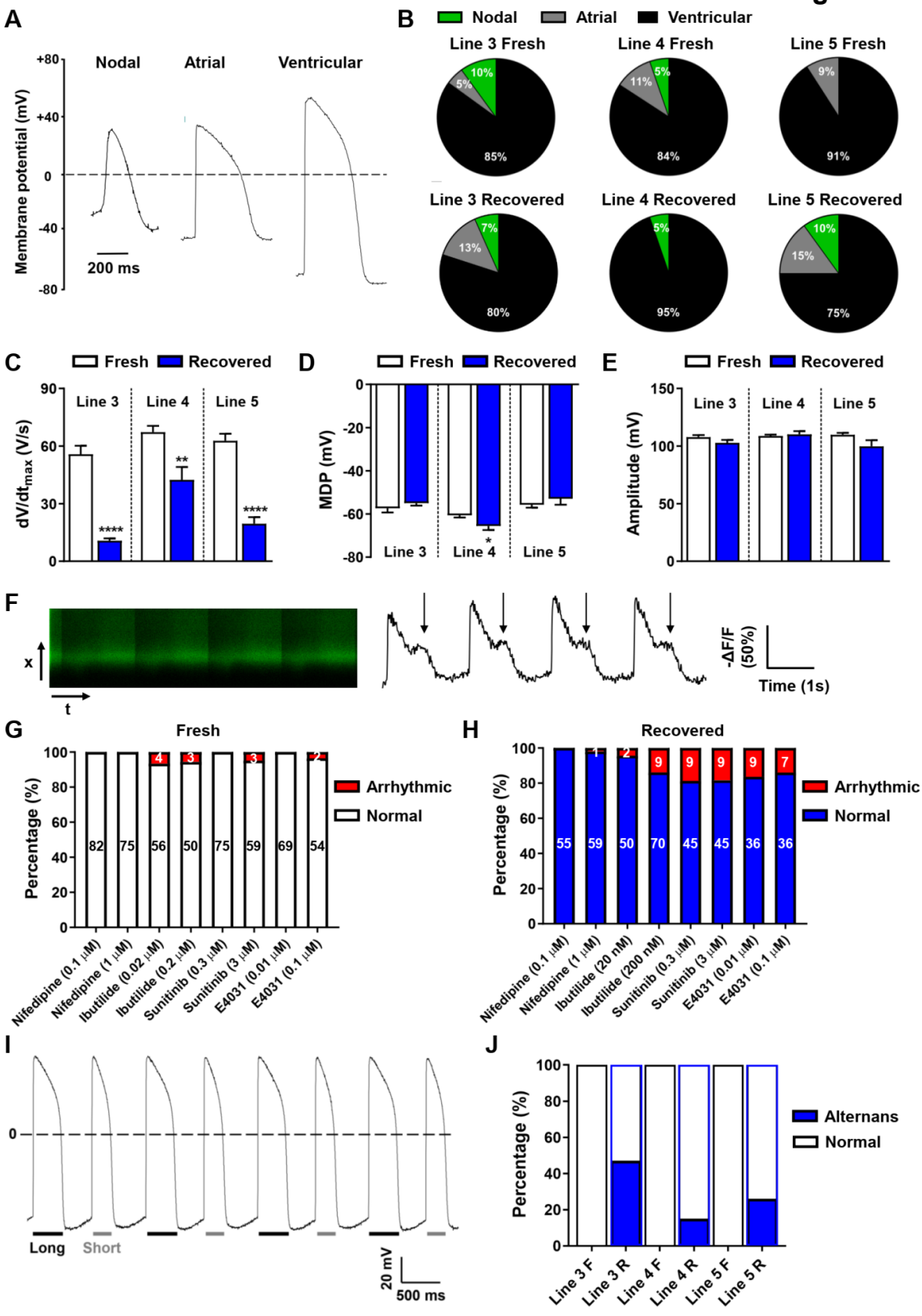

A
Vehicle
Nifedipine (0.1 μM)
Fresh
0
Recovered
0
0.1 mV
1 s
B
Vehicle
Ibutilide (0.02 μM)
Fresh
0
Recovered
0
0.1 mV
1 s
C
Vehicle
Sunitinib (0.3 μM)
Fresh
0
Recovered
0
0.1 mV
1 s
D
Vehicle
E4031 (0.01 μM)
Fresh
0
Recovered
0
0.1 mV
1 s
E
Triggered activity
0
0.2 mV
1 s
F
EADs
0
0.2 mV
1 s

Figure S6


A
Nodal
Atrial
Ventricular
Membrane potential (mV)
+80
+40
0
-40
-80
200 ms
B
Nodal
Atrial
Ventricular
Line 3 Fresh
Line 4 Fresh
Line 5 Fresh
Line 3 Recovered
Line 4 Recovered
Line 5 Recovered
10%
5%
85%
5%
11%
84%
9%
91%
7%
13%
80%
5%
95%
10%
15%
75%
C
Fresh
Recovered
dV/dtmax (V/s)
Line 3
Line 4
Line 5
****
**
****
D
Fresh
Recovered
MDP (mV)
Line 3
Line 4
Line 5
*
E
Fresh
Recovered
Amplitude (mV)
Line 3
Line 4
Line 5
F
x
t
-ΔF/F (50%)
Time (1s)
G
Fresh
Percentage (%)
Arrhythmic
Normal
82
75
56
50
75
59
69
54
4
3
3
2
Nifedipine (0.1 μM)
Nifedipine (1 μM)
Ibutilide (0.02 μM)
Ibutilide (0.2 μM)
Sunitinib (0.3 μM)
Sunitinib (3 μM)
E4031 (0.01 μM)
E4031 (0.1 μM)
H
Recovered
Percentage (%)
Arrhythmic
Normal
55
59
50
70
45
45
36
36
1
2
9
9
9
9
7
Nifedipine (0.1 μM)
Nifedipine (1 μM)
Ibutilide (20 nM)
Ibutilide (200 nM)
Sunitinib (0.3 μM)
Sunitinib (3 μM)
E4031 (0.01 μM)
E4031 (0.1 μM)
I
0
Long
Short
20 mV
500 ms
J
Percentage (%)
Alternans
Normal
Line 3 F
Line 3 R
Line 4 F
Line 4 R
Line 5 F
Line 5 R

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Flow cytometry analysis of cardiac troponin T positive (cTnT+) hiPSC-CMs and cell viability, Related to Figure 1. Flow cytometry showed that over 90% of cells were cTNT+ cardiomyocytes in both **(A)** fresh and **(B)** recovered hiPSC-CMs. **(C)** Representative flow cytometry plot of live/dead analysis of hiPSC-CMs after 6-month (left) or 18-month (right) cryopreservation. Cells were thawed and immediately analyzed using calcein and ethidium homodimer-1 (EthD-1). Quantification of the percentage of **(D)** live and **(E)** dead cells after 6-month or 18-month cryopreservation (n=6 per group from Lines 1-3).

Figure S2. Characterization of gene expression, proliferation, and cell viability of fresh and recovered hiPSC-CMs, Related to Figures 1 and 3. (A) Genes associated with electrophysiology, Ca^{2+} handling, and myofilaments showed comparable expression between fresh and recovered hiPSC-CMs (n=3 lines, Lines 1-3). **(B-C)** Protein expression of CONNEXIN 40 encoded by *GJA5* was comparable between fresh and recovered groups (n=3 lines, Lines 1-3). **(D)** *MHY7/MYH6* Ratio was comparable between fresh and recovered hiPSC-CMs (n=3 lines, Lines 1-3). **(E-F)** Representative immunostaining images of BrdU indicated the increased number of proliferative cells in recovered hiPSC-CMs compared with fresh hiPSC-CMs. Scale bars represent 100 μm. **(G)** Quantification of BrdU positive hiPSC-CMs (n=3 lines, Lines 1-3). Six randomly selected regions per cell line were imaged for each group. **(H)** Representative fluorescent images calcein-loaded hiPSC-CMs on MEA recording electrodes showing the comparable calcein fluorescence. **(I)** Measurement of calcein fluorescence intensity (n=12 from Line 1). **(J)** Measurement of cell viability using the ATP-based luminescent assay (n=12 from Line 1). All data represented as mean

± SEM, *p<0.05 and **p<0.01 compared with their respective fresh samples. L, Line; F, Fresh; R, Recovered.

Figure S3. Drug response of Line 2 fresh and recovered hiPSC-CMs, Related to Figure 4. Drug response of fresh and recovered hiPSC-CMs treated with **(A-B)** Nifedipine [Fresh, n=32 (0.01 μM), n=37 (0.1 μM); Recovered, n=23 (0.01 μM), n=24 (0.1 μM)], **(C-D)** Ibutilide [Fresh, n=29 (0.02 μM), n=20 (0.2 μM); Recovered, n=16 (0.02 μM), n=25 (0.2 μM)], **(E-F)** Sunitinib [Fresh, n=43 (0.3 μM), n=27 (3 μM); Recovered, n=15 (0.3 μM), n=18 (3 μM)], and **(G-H)** E4031 [Fresh, n=31 (0.01 μM), n=22 (0.1 μM); Recovered, n=14 (0.01 μM), n=14 (0.1 μM)]. Raw APD50 and APD90 data were presented in panels **(A)**, **(C)**, **(E)**, and **(G)**. Data of relative change to vehicle were presented in panels **(B)**, **(D)**, **(F)**, and **(H)**. The cell number of vehicle was 60 and 47 in fresh and recovered groups, respectively and the same dataset was used for plotting graphs in four drug treatments. Data were collected from three independent dish preparations from two independent differentiations for each line. All data represented as mean ± SEM, **p<0.01, ***p<0.001, and ****p<0.0001 compared with their respective fresh samples at the same dose.

Figure S4. Drug response of Line 3 fresh and recovered hiPSC-CMs, Related to Figure 4. Drug response of fresh and recovered hiPSC-CMs treated with **(A-B)** Nifedipine [Fresh, n=17 (0.01 μM), n=19 (0.1 μM); Recovered, n=14 (0.01 μM), n=14 (0.1 μM)], **(C-D)** Ibutilide [Fresh, n=13 (0.02 μM), n=15 (0.2 μM); Recovered, n=11 (0.02 μM), n=15 (0.2 μM)], **(E-F)** Sunitinib [Fresh, n=19 (0.3 μM), n=17 (3 μM); Recovered, n=12 (0.3 μM), n=12 (3 μM)], and **(G-H)** E4031 [Fresh, n=16 (0.01 μM), n=19 (0.1 μM); Recovered, n=10 (0.01 μM), n=11 (0.1 μM)]. Raw APD50 and APD90 data were presented in panels **(A)**, **(C)**, **(E)**, and **(G)**. Data of relative change

to vehicle were presented in panels **(B)**, **(D)**, **(F)**, and **(H)**. The cell number of vehicle was 18 and 21 in fresh and recovered groups, respectively and the same dataset was used for plotting graphs in four drug treatments. Data were collected from three independent dish preparations from two independent differentiations for each line. All data represented as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 compared with their respective fresh samples at the same dose.

Figure S5. Representative MEA recordings Related to Figure 5. Raw MEA traces of fresh hiPSC-CMs from Line 1 treated with **(A)** Nifedipine (0.1 μM), **(B)** Ibutilide (0.02 μM), **(C)** Sunitinib (0.3 μM), and **(D)** E4031 (0.01 μM). Fresh hiPSC-CMs showed the expected pharmacological response to drug treatment. **(E)** Representative MEA trace of triggered activity (red box) in Line 2 recovered hiPSC-CMs treated with Sunitinib (3 μM). **(F)** Representative MEA trace of early after-depolarizations (EADs) (red triangles) in Line 5 recovered hiPSC-CMs treated with Ibutilide (0.2 μM).

Figure S6. Recovered hiPSC-CMs are more susceptible to drug-induced arrhythmic events, Related to Figure 6. (A) Action potential morphology of hiPSC-CMs was examined by the single-cell patch clamp. **(B)** Proportion of cardiomyocyte subtypes in Lines 3-5 hiPSC-CMs [Line 3 Fresh, n=17 (Ventricular), n=1 (Atrial), and n=2 (Nodal); Line 3 Recovered, n=12 (Ventricular), n=2 (Atrial), and n=1 (Nodal); Line 4 Fresh, n=16 (Ventricular), n=2 (Atrial), and n=1 (Nodal); Line 4 Recovered, n=18 (Ventricular) and n=1 (Nodal); Line 5 Fresh, n=20 (Ventricular) and n=2 (Atrial); Line 5 Recovered, n=15 (Ventricular), n=3 (Atrial), and n=2 (Nodal)]. Quantification of **(C)** maximum dV/dt, **(D)** maximum diastolic potential (MDP), and **(E)** amplitude of action potential in fresh and recovered hiPSC-CMs (Line 3 Fresh, n=16; Line 3 Recovered, n=12; Line 4

Fresh, n=15; Line 4 Recovered, n=18; Line 5 Fresh, n=19; Line 5 Recovered, n=15). Data were collected from four independent dish preparations from one differentiation. **(F)** Representative arrhythmic events in Line 1 recovered hiPSC-CMs treated with 0.3 μM Sunitinib using ASAP2. Arrows indicate EADs. **(G)** The percentage of fresh hiPSC-CMs with arrhythmic events in Lines 1-3 hiPSC-CMs. **(H)** The percentage of recovered hiPSC-CMs with arrhythmic events in Lines 1-3 hiPSC-CMs. Arrhythmic events include EADs, delayed afterdepolarizations (DADs), and irregular beating. Cardiac arrest is not included in the analysis. Data were collected from three independent dish preparations from two independent differentiations for each line. Numbers indicate cell number under each condition. **(I)** Action potential morphology of alternans in Line 5 recovered hiPSC-CMs. **(J)** The percentage of cells exhibiting alternans in Lines 3-5 hiPSC-CMs. All data represented as mean ± SEM, *$p<0.05$, **$p<0.01$, and ****$p<0.0001$ compared with their respective fresh samples. F, Fresh; R, Recovered.

SUPPLEMENTARY TABLES

Table S1. List of differentially expressed genes (DEGs), Related to Figure 1.

Table S2. Classification criteria for cardiomyocyte subtypes, Related to Figure S6.

Parameter	Value	Classification
APD30-40/APD70-80	<1.5 >1.5	**Nodal / Atrial** **Ventricular**
APD50/APD90	<0.7 >0.7	**Nodal / Atrial** **Ventricular**
Mean dV/dt_{max}	<0.3 0.3-10 >10	**Nodal** **Atrial** **Ventricular**
Mean amplitude	<75 75-90 >90	**Nodal** **Atrial** **Ventricular**
MDP	<45 45-50 >50	**Nodal** **Atrial** **Ventricular**
APD: action potential duration **MDP:** maximum diastolic potential		

Table S3. Drug-induced arrhythmic events in MEA measurements, Related to Figure 6.

Nifedipine (μM)						
Line	**Fresh**			**Recovered**		
	0.01	0.1	1	0.01	0.1	1
1	0	0	0	0	Tact 3/6	Tact 6/6
2	0	0	0	0	0	0
3	0	0	0	0	Arrest 3/6	Arrest 5/6
4	0	0	0	0	0	Tact 2/6
5	0	0	0	0	0	Arrest 6/6
Ibutilide (μM)						
Line	**Fresh**			**Recovered**		
	0.002	0.02	0.2	0.002	0.02	0.2
1	0	0	0	0	0	0
2	0	0	EADs 3/6	0	EADs 4/6	Arrest 6/6
3	0	0	0	0	EADs 4/6	EADs 5/6
4	0	0	0	0	0	0
5	0	0	EADs 5/6	0	EADs 5/6	EADs 5/6
Sunitinib (μM)						
Line	**Fresh**			**Recovered**		
	0.03	0.3	3	0.03	0.3	3
1	0	0	0	0	0	0
2	0	0	0	0	0	Tact 2/6
3	0	0	0	0	0	EADs 2/6
4	0	0	0	0	0	EADs 2/6
5	0	0	0	EADs 2/6	EADs 3/6	EADs 1/6
E4031 (μM)						
Line	**Fresh**			**Recovered**		
	0.001	0.01	0.1	0.001	0.01	0.1
1	0	0	0	0	0	0
2	0	0	0	0	0	Tact 1/6
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	EADs 3/6

Tact: Triggered activity
EADs: Early after-depolarizations
Arrest: No electrical signal
0: No arrhythmic event, the normal measurements were 6.
x/6: Number of MEA measurements positive for arrhythmic events
The remaining (6-x) measurements were normal.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Reprograming and maintenance of hiPSCs in culture. Five hiPSC lines (SCVI273, SCVI116, SCVI202, SCVI15, SCVI34) from healthy donors without known cardiovascular disease were provided by Stanford Cardiovascular Institute (CVI) Biobank. Briefly, hiPSCs were reprogramed from human peripheral blood mononuclear cells (PBMCs) using the CytoTune^{TM}-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific) cultured on feeder-free Matrigel (Fisher Scientific)-coated culture plates (E&K Scientific). Established hiPSCs exhibited positive immunostaining for the stem cell markers and were further characterized by running single nucleotide polymorphism (SNP) (Illumina Infinium OmniExpress-24 Kit) for both PBMCs and hiPSCs. Karyotyping demonstrated stable chromosomal integrity in hiPSC lines (data are available from Stanford CVI Biobank). HiPSCs were routinely maintained in the E8 medium (GIBCO, Life Technologies). When hiPSCs reached a confluency of 70%-80%, hiPSCs were dissociated by 0.5 mM EDTA at 37°C for 6 min, followed by centrifugation at 300× g for 3 min at room temperature. Cells were then re-suspended in the E8 medium supplemented with 10 μM Y-27632 ROCK inhibitor (MedChem Express) for 24 hr. A medium change was performed every 24 hr. Cultures were maintained at 37°C in a humidified incubator with 5% CO_2 and 5% O_2.

Fluorescence-activated cell sorting (FACS) analysis. Day 23 fresh or 1-week recovered hiPSC-CMs were used for the analysis. Collected cell pellets were washed once in ice-cold phosphate-buffered saline (PBS; pH 7.4) followed by flow cytometric staining using the BD flow cytometry analysis protocol for intracellular markers. Briefly, cells were fixed and permeabilized by BD Cytofix/Cytoperm for 20 min at 4°C (BD Biosciences). After washing in BD Perm/Wash buffer, cells were incubated with a rabbit anti-cardiac troponin T (cTNT) antibody (Ab45932, Abcam;

1:200) or rabbit IgG isotype control (02-6102, Thermo Fisher Scientific) with the same dilution for 60 min at room temperature. Cells were washed once and then incubated with a goat anti-rabbit Alexa Fluor-488 (A11034, Thermo Fisher Scientific; 1:250) for 30 min at 4°C. Following a wash, cells were re-suspended in the FACS buffer consisting of 1% fetal bovine serum (FBS) and 1 mM EDTA in PBS. Cells were analyzed by a BD Biosciences FACS Aria II instrument fitted with a 100 μm nozzle using FACSDiva software.

For live/dead analysis of live cells, hiPSC-CMs that had been frozen for 6 or 18 months were thawed as described above. Live/dead staining was conducted following the protocol of live/dead viability/cytotoxicity kit for mammalian cells (Thermo Fisher Scientific). Briefly, thawed cells were re-suspended in RPMI/B27 supplemented with 5% KOSR and were incubated with 0.1 μM calcein AM and 8 μM ethidium homodimer-1 for 15 min at room temperature, and protected from light. After incubation, cells were analyzed by a BD Biosciences FACS Aria II instrument fitted with a 100 μm nozzle using FACSDiva software.

Immunostaining and imaging. For 5-Bromo-2'-deoxyuridine (BrdU) staining, hiPSC-CMs were incubated with BrdU (10 μM) (Sigma Aldrich) overnight at 37°C. Cells were fixed in 4% paraformaldehyde (PFA) (Thermo Fisher Scientific) for 10 min and permeabilized with 0.2% Triton X-100 (Sigma Aldrich) in PBS (Thermo Fisher Scientific) for 1 hr at room temperature. DNA was denatured by 2 M HCl (Sigma Aldrich) for 10 min at 37°C followed by neutralization with 0.1 M Borate buffer (pH 8.3) (EMD Millipore) for 5 min twice at room temperature. Cells were then blocked with 3% bovine serum albumin (BSA) (Sigma Aldrich) in PBS for 1 hr, followed by overnight incubation with primary antibodies to cTNT (Ab45932, Abcam), α-ACTININ (A7811, Sigma Aldrich), or BrdU (14-5071-82, Thermo Fisher Scientific) at 1:100

dilution in PBS with 1% BSA at 4°C. Cells were washed three times with 0.2% Tween-20 (Sigma Aldrich) PBS solution and then incubated for 1 hr at room temperature in the dark with goat anti-mouse or anti-rabbit immunoglobulin G conjugated to Alexa Fluor 488 (A11001, Thermo Fisher Scientific; 1:200) or 594 secondary antibodies (A11012, Thermo Fisher Scientific; 1:200). Cells were washed again three times as described above, mounted with ProLong® Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). Fluorescent images were captured on a Zeiss LSM 710 confocal microscope equipped with an oil immersion objective (63×, NA: 1.4) or Leica DMi 8 fluorescence microscope with an objective (20x, NA: 0.4). Image processing and analysis were performed using ImageJ.

Cell viability assay. Cells were loaded with calcein (1 μM) at room temperature for 20 min. Fluorescent images of calcein were taken using Cytation 5 (Biotek) automated imaging mode. The fluorescence of calcein was measured using Cytation 5 microplate reader mode. For the ATP-based cell viability assay, according to the manufacturer's protocol (Promega), a volume of CellTiter-Glo® Reagent equal to the volume of cell culture medium present in each well was added followed by incubation at room temperature for 10 min. Luminescence was measured using Cytation 5 microplate reader mode.

Ca^{2+} imaging. Intracellular Ca^{2+} imaging was performed as previously described (Ma et al., 2018). Briefly, hiPSC-CMs that were grown on Matrigel-coated glass coverslips were loaded with the cell-permeable Ca^{2+} sensitive dye Fura-2 AM (2 μM) in 1.8 mM Ca^{2+}-Tyrode's solution with 0.1% F-127 (Life Technology) for 10 min. Tyrode's solution contains 135 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, and 10 mM HEPES (pH 7.4 with NaOH). After 5 min of incubation

in 1.8 mM Ca^{2+} Tyrode's solution to allow de-esterification, coverslips were mounted on the stage of an inverted epifluorescence microscope (Nikon Eclipse Ti-S) with 40× oil immersion objective (NA: 0.95). Cardiomyocytes were field stimulated at 0.5 Hz with a pulse duration of 10 ms. Fura-2 AM-loaded cells were excited with the Lambda DG-4 ultra-high speed wavelength switching light source (Sutter Instrument) at both 340 and 380 nm, and the emission fluorescence signal was collected at 510 nm with iXon Ultra 897 EMCCD (Andor). Changes in fluorescence signal of single isolated hiPSC-CM were measured by the NIS Elements AR software, which permits the recording of multiple cells. Intracellular Ca^{2+} dynamics were expressed as changes in the ratio of F340/F380.

Lentiviral vector infection and optical imaging of ASAP2. Optical imaging of action potential using voltage sensor Allosteric Sensor for Action Potentials 2 (ASAP2) was performed as previously described (Zhang et al., 2019). hiPSC-CMs were seeded on a Matrigel-coated 35 mm glass-bottom dish with 20 mm micro-well (Cellvis) and infected with pLLM-ASAP2 lentivirus with the multiplicity of infection (MOI) of three in the RPMI-B27 medium. The medium containing lentivirus was replaced with fresh RPMI-B27 24 hr after infection. Optical imaging of ASAP2 in single hiPSC-CMs was performed three days after infection. Cells were maintained in 1.8 mM Ca^{2+} Tyrode's solution at 37°C during recording. ASAP2 was excited at 488 nm and emission was collected over 510 nm. Line scan images were acquired on a Zeiss LSM710 confocal microscope (Zeiss) equipped with a 20× objective (NA: 0.8). Line scan images were processed to enhance brightness for presentation purposes using ImageJ. Raw imaging data were analyzed using a custom-written MATLAB program. ASAP2 data were presented as $-\Delta F/F$. For drug-testing experiments, two concentrations of each drug were studied. Concentrated (2×) testing solutions

for each concentration were prepared freshly on the day of experiments by diluting DMSO stocks into 1.8 mM Ca^{2+} Tyrode's solution. The targeted concentration was attained when drugs were added to the imaging dishes. The same amount of DMSO was used as vehicle control.

Patch clamp recordings. Electrophysiological properties of each line were recorded using an EPC-10 patch clamp amplifier (HEKA, Germany). hiPSC-CMs were enzymatically dissociated using TrypLE™ Select Enzyme (10X) (Thermo Fisher Scientific) for 6 min at 37°C and were seeded in 35 mm petri dishes coated with Matrigel (Corning). All recordings were performed at 37ºC using a chamber (Warner instrument, USA) mounted on an inverted microscope (Nikon, Japan). Cells were maintained in 1.8 mM Ca^{2+} Tyrode's solution during recording. Thin-wall glass filaments were pooled to obtain 2.5-4 MΩ pipettes using a micropipette puller (Sutter Instrument, USA). Five parameters were used as clarification criteria for determining cardiomyocyte subtypes (**Table S2**). Data plotting and statistics were conducted using GraphPad Prism software.

Immunoblotting. Total proteins were extracted from fresh and recovered hiPSC-CMs using RIPA buffer (Thermo Fisher Scientific) supplemented with a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Each sample was subjected to electrophoresis on 4–12% NuPAGE Bis-Tris gradient gels (Thermo Fisher Scientific), and proteins were transferred to PVDF membranes (Thermo Fisher Scientific). Membranes were incubated overnight with rabbit CONNEXIN 40 antibody (36-4900, Thermo Fisher Scientific; 1:500), followed by incubation for 1 hr with anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (7074S, Cell Signaling Technology; 1:5000). The housekeeping protein GAPDH was blotted by incubating 1 hr with the GAPDH-loading control antibody conjugated with HRP (MA515738HRP, Thermo Fisher

Scientific; 1:10,000). Signals were detected using the SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific). Blot intensity was quantified using Image Lab 5.2.1 (Biorad).

RNA extraction and quantitative polymerase chain reaction (qPCR). Total RNA from day 30 fresh and recovered hiPSC-CMs was extracted and purified using a miRNeasy Micro Kit (QIAGEN), according to the manufacturer's instruction. The quantity and quality of RNA were determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). RNA was then reversely transcribed using the iScript™ cDNA synthesis kit (Biorad) according to the manufacturer's instructions. The qPCR was performed using TaqMan® Universal PCR Master Mix (Fisher Scientific) and Taqman probes (Life Technologies) on a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific) in 20 μL reaction. Relative mRNA levels were normalized to those of 18S mRNA in each reaction. Three biological replicates per group were used for qPCR.

RNA-sequencing and analysis. Library preparations were conducted using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs) and subjected to sequencing on a Hiseq 4000 platform (Novogene). For RNA-seq data, the quality was examined by way of analyzing per base sequence quality plots using FastQC. The trimming of sequence reads was done by TrimGalore. RNA-seq reads were aligned to the human genome (hg38) using the STAR software (Dobin et al., 2013) and a gene database constructed from ENCODEv31 (Rosenbloom et al., 2012). Reads that overlapped with exon coordinates were counted using RSEM and featureCounts (Li and Dewey, 2011; Liao et al., 2014). Raw read counts were transformed using the variance stabilizing transformation (VST) function included in the DESeq2 R package. Mean and standard deviations of normalized expressions were calculated for each gene. Z-scores were

determined by subtracting the mean from each expression value and dividing by the standard deviation. Differentially expressed genes (DEGs) between different groups were identified using the DESeq2 R package (Anders et al., 2012). Genes with a Benjamin-Hochberg corrected $p<0.05$ were considered significant. DEGs were classified into the categories of biological process, cellular component, and molecular function using gene ontology annotation. The metabolic pathways of the DEGs were predicted using the Kyoto Encyclopedia of Genes and Genomes (KEGG). To identify potential common pathways, DEGs were analyzed for biological process and pathway enrichment using DAVID and PANTHER (Huang da et al., 2009; Mi et al., 2013). Pathway enrichment analysis was performed to identify significantly enriched metabolic pathways or signal transduction pathways using the $FDR<0.05$ as a threshold of significance.

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