Supplementary Data

Cardiac Toxicity from Ethanol Exposure in Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes

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Supplementary Methods

The effect of ROS scavenger N-acetyl cysteine (NAC) on the ethanol-induced phenotype. hiPSC-CMs were dissociated using 0.25% trypsin-EDTA and plated onto Matrigel-coated 96-well culture plates at a density of $2.5x10^4$ cells/well and cultured for 2 days to allow the cells to recover spontaneous beating. The cells were pre-treated with ROS scavenger NAC (Sigma) at 2 mM for 1 h and then treated with 0 or 50 mM ethanol in the presence of 2 mM NAC for 5 days. The dose of NAC was selected based on previous studies showing that 2 mM NAC was effective in reducing ROS production in cells treated with substances that stimulate oxidative stress (Yiran *et al.*, 2013; Zhen *et al.*, 2015). hiPSC-CMs treated with 0 or 50 mM ethanol without NAC were used as controls. The cells were covered with mineral oil to prevent ethanol evaporation, and medium was changed daily. The amount of ROS produced by ethanol exposure and its effects on Ca^{2+} transients were evaluated at the end of the treatment.

The generation of intracellular and mitochondrial ROS was assessed using carboxy-H2DCFDA (Thermo Fisher Scientific) and MitoSOX Red (Thermo Fisher Scientific), respectively. The ethanol-containing medium and mineral oil were aspirated and cells were washed twice with warm D-PBS and incubated with both 25 µm carboxy-H2DCFDA and 5 µM MitoSOX Red working solution in warm D-PBS for 15 min at 37ºC, protected from light. Cells were washed twice with warm D-PBS and counter-stained with Hoechst (Thermo Fisher Scientific) in warm buffer and imaged immediately using an ArrayScan™ XTI Live High Content Platform (Life Technologies). Images of carboxy-H2DCFDA, MitoSOX Red and Hoechst were acquired and quantitatively analyzed using ArrayScan™ XTI Live High Content Platform. Twenty fields/well were selected and 4 replicate wells per condition were imaged using a 10x objective. Acquisition software Cellomics Scan (Thermo Fisher Scientific) was used to capture images, and data analysis were performed using Cellomics View Software (Thermo Fisher Scientific). Images were analyzed with mask modifier for Hoechst restricted to the nucleus. Carboxy-H2DCFDA and MitoSOX Red were each quantified with a spot mask that extended 7 units from the nucleus. Spot threshold was set to 10 units and detection limit was set at 25 units. Mean carboxy-H2DCFDA and MitoSOX Red average fluorescence intensity of cells per well in each treatment group was used as readout.

Live cell imaging of intracellular Ca^{2+} transient was performed using Fluo-4, AM, a cell permeant-fluorescent Ca^{2+} dye (Thermo Fisher Scientific). Cells were incubated with the 10 μM Fluo-4, AM for 20 min at 37°C followed by a 20 min wash at room temperature in Tyrode's solution (148 mM NaCl, 4 mM KCl, 0.5 mM $MgCl_2 \cdot 6H_2O$, 0.3 mM $NaPH_2O_4 \cdot H_2O$, 5 mM HEPES, 10 mM D-Glucose, 1.8 mM $CaCl_2 \cdot H_2O$, pH adjusted to 7.4 with NaOH). Fluorescence was imaged over time using an ImageXpress Micro XLS System (Molecular Devices) at 20x objective and 30 frame per second. Fluorescence was measured from the entire cell region and dye excitation at 488 nm and emission at >500 nm.

References

Jha, R., Wu, Q., Singh, M., Preininger, M. K., Han, P., Ding, G., Cho, H. C., Jo, H., Maher, K. O., Wagner, M. B.*, et al.* (2016). Simulated microgravity and 3D culture enhance induction, viability, proliferation and differentiation of cardiac progenitors from human pluripotent stem cells. *Sci Rep* **6**, 30956.

Yiran, Z., Chenyang, J., Jiajing, W., Yan, Y., Jianhong, G., Jianchun, B., Xuezhong, L., and Zongping, L. (2013). Oxidative stress and mitogen-activated protein kinase pathways involved in cadmium-induced BRL 3A cell apoptosis. *Oxid Med Cell Longev* **2013**, 516051.

Zhen, Y. Z., Lin, Y. J., Li, K. J., Yang, X. S., Zhao, Y. F., Wei, J., Wei, J. B., and Hu, G. (2015). Gambogic acid lysinate induces apoptosis in breast cancer mcf-7 cells by increasing reactive oxygen species. *Evid Based Complement Alternat Med* **2015**, 842091.

Supplementary Table 1. Stages of alcohol intoxication

Blood alcohol concentration (BAC) in g/100 ml of blood	**Corresponding concentration in mM**	**Stage**
0.03 - 0.12% 0.08%: Legally permissible limit	17 mM	euphoria
0.09 - 0.25%	50 mM	excitement
0.18 - 0.30%	100 mM	confusion
> 0.45%	200 mM	death

Adapted from http://www.intox.com/t-physiology.aspx

Supplementary Table 2. Primary antibodies for immunocytochemistry

Target	**Isotype**	**Supplier**	**Catalog #**	**Dilution**
α-actinin	mouse IgG_1	Sigma	A7811	1:800
NKX2-5	rabbit IgG	Santa Cruz Biotech	SC14033	1:200
cardiac troponin T	mouse IgG_1	Fisher Scientific	MS295P1	1:400
cardiac troponin I	mouse IgG_{2b}	Millipore	MAB1691	1:200
pan cadherin	mouse IgG_1	Sigma	C1821	1:200

Supplementary Table 3. SyBr green primers for qRT-PCR

Gene	Full name (other name)	Accession code	Primer
ACTN1	Actinin, alpha 1 (ACTN1), transcript variant 3, mRNA	NM_001102	Forward: AGGTGGGAGTTACACCATGC Reverse: ACATGCAGCCAGAAGAGGAC
cTnI	Troponin I type 3	NM_000363	Forward: CTCAAACTTTTTCTTGCGGC, Reverse: GTGAAGAAGGAGGACACCGA
TNNT2	Troponin T type 2	NM_001001431	Forward: GCGGGTCTTGGAGACTTTCT Reverse: TTCGACCTGCAGGAGAAGTT
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_001256799	Forward: CTGGGCTACACTGAGCACC Reverse: AAGTGGTCGTTGAGGGCAATG

Note: Primers were retrieved from Jha *et al*., 2016 and http://pga.mgh.harvard.edu/primerbank/

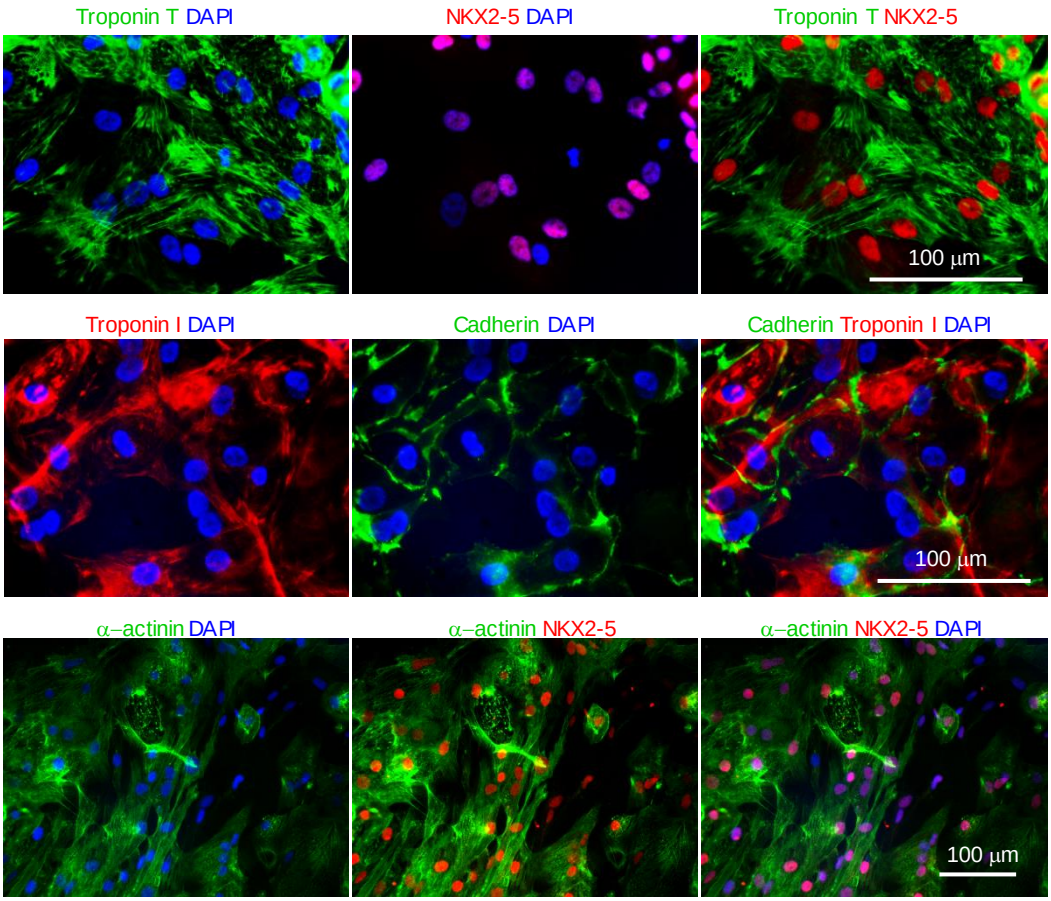
Supplementary Table 4. List of top 60 genes significantly altered in hiPSC-CMs treated with 50mM ethanol compared with untreated cells

Symbol	**RefseqID**	**Gene name**	**Fold change**	**Adjusted P-value**
Up-regulated				
RN7SL1	NR_002715.1	RNA, 7SL, cytoplasmic 1	2.29	1.19E-02
MMP9	NM_004994.2	matrix metallopeptidase 9	2.17	4.58E-07
FAM19A4	NM_182522.4	family with sequence similarity 19 member A4, C-C motif chemokine like	1.56	5.21E-03
EMID1	NM_133455.3	EMI domain containing 1	1.53	5.58E-03
COL14A1	NM_021110.3	collagen type XIV alpha 1 chain	1.51	2.15E-12
LOC100049716	NR_122124.1	uncharacterized LOC100049716	1.50	3.64E-02
SYT10	NM_198992.3	synaptotagmin 10	1.49	1.60E-03
HMCN1	NM_031935.2	hemicentin 1	1.49	2.55E-02
SCIN	NM_001112706.2	scinderin	1.49	4.75E-03
EDNRB	NM_000115.4	endothelin receptor type B	1.48	4.26E-02
FREM2	NM_207361.5	FRAS1 related extracellular matrix protein 2	1.48	1.67E-02
KCNIP2	NM_014591.4	potassium voltage-gated channel interacting protein 2	1.47	2.06E-07
TRH	NM_007117.4	thyrotropin releasing hormone	1.44	5.43E-05
TF	NM_001063.3	transferrin	1.43	9.90E-05
SLC16A14	NM_152527.4	solute carrier family 16 member 14	1.42	6.80E-03

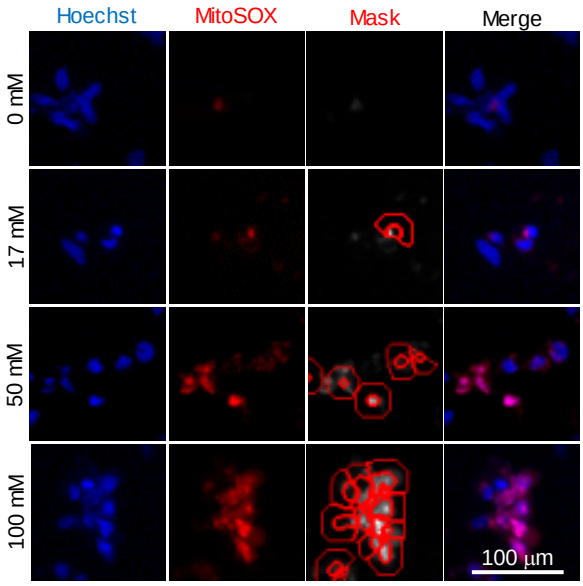
KIT	NM_000222.2	KIT proto-oncogene receptor tyrosine kinase	1.41	3.72E-03
PEG10	NM_001184962.1	paternally expressed 10	1.41	4.56E-07
ARRDC4	NM_183376.2	arrestin domain containing 4	1.38	9.90E-04
RASL12	NM_016563.3	RAS like family 12	1.38	3.30E-02
DIRAS3	NM_004675.3	DIRAS family GTPase 3	1.37	1.78E-03
SLC5A3	NM_006933.6	solute carrier family 5 member 3	1.36	1.63E-03
KCNG1	NM_002237.3	potassium voltage-gated channel modifier subfamily G member 1	1.36	3.89E-03
RPLP2	NM_001004.3	ribosomal protein lateral stalk subunit P2	1.34	3.28E-04
KCTD12	NM_138444.3	potassium channel tetramerization domain containing 12	1.34	4.37E-03
B4GAT1	NM_006876.2	beta-1,4-glucuronyltransferase 1	1.34	1.78E-03
RXRG	NM_006917.4	retinoid X receptor gamma	1.33	2.24E-02
SMOC1	NM_001034852.2	SPARC related modular calcium binding 1	1.33	1.51E-03
HSPE1	NM_002157.2	heat shock protein family E (Hsp10) member 1	1.33	4.63E-03
WNT2	NM_003391.2	Wnt family member 2	1.33	3.63E-02
ASTN1	NM_004319.2	astrotactin 1	1.32	1.81E-05
Down-regulated				
ANKRD1	NM_014391.2	ankyrin repeat domain 1	0.23	1.03E-05
ATF3	NM_001674.3	activating transcription factor 3	0.34	2.26E-05

CYR61	NM_001554.4	cysteine rich angiogenic inducer 61	0.35	5.91E-51
CTGF	NM_001901.2	connective tissue growth factor	0.37	3.82E-33
FCRLA	NM_001184866.1	Fc receptor like A	0.45	7.98E-04
GC	NM_000583.3	vitamin D binding protein	0.47	4.56E-08
NPPB	NM_002521.2	natriuretic peptide B	0.47	5.48E-07
GADD45G	NM_006705.3	growth arrest and DNA damage inducible gamma	0.47	1.63E-05
TMEM71	NM_144649.2	transmembrane protein 71	0.47	1.53E-05
LOC100506725	NR_108082.1	uncharacterized LOC100506725	0.50	4.50E-04
CYP2C9	NM_000771.3	cytochrome P450 family 2 subfamily C member 9	0.50	4.70E-04
DNAAF3	NM_001256714.1	dynein axonemal assembly factor 3	0.50	1.21E-02
SPP1	NM_001040058.1	secreted phosphoprotein 1	0.51	9.03E-05
SLC5A1	NM_000343.3	solute carrier family 5 member 1	0.51	1.74E-03
LMOD2	NM_207163.2	leiomodin 2	0.52	4.64E-07
CFH	NM_000186.3	complement factor H	0.52	8.05E-04
SLC17A4	NM_005495.2	solute carrier family 17 member 4	0.54	2.41E-02
MYH4	NM_017533.2	myosin heavy chain 4	0.54	5.70E-04
MYL2	NM_000432.3	myosin light chain 2	0.54	4.87E-04
UGT2A3	NM_024743.3	UDP glucuronosyltransferase family 2 member A3	0.55	2.28E-04
TNFRSF12A	NM_016639.2	TNF receptor superfamily member 12A	0.56	2.26E-11

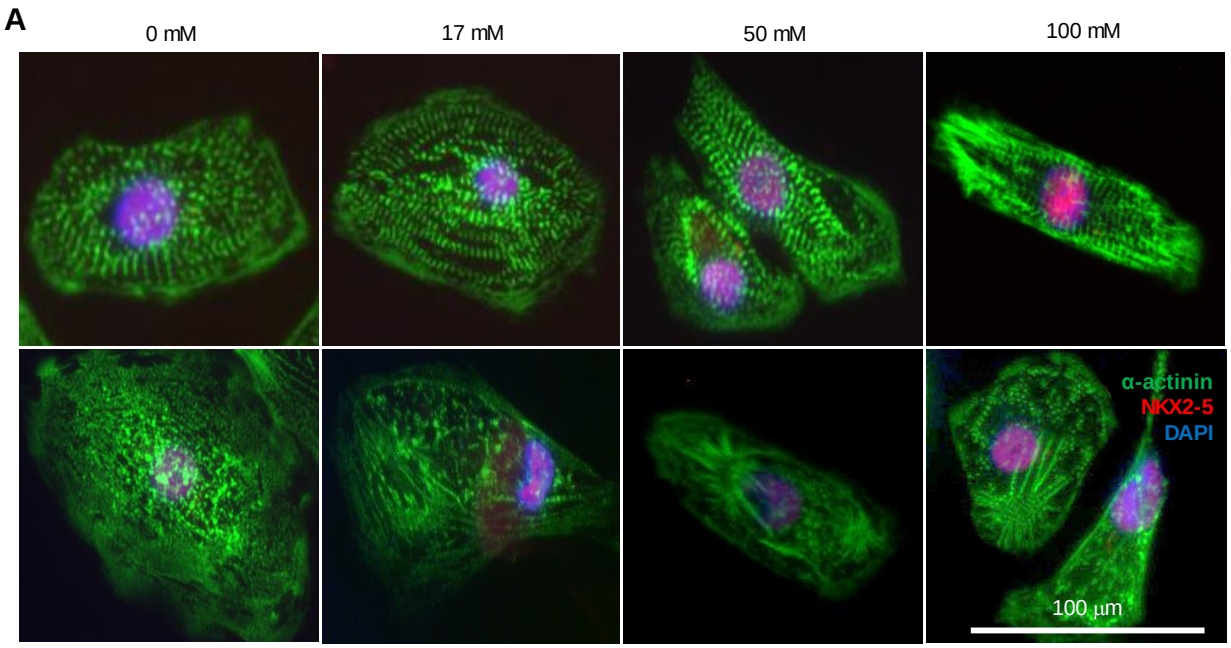
OTUD1	NM_001145373.2	OTU deubiquitinase 1	0.57	7.57E-18
KCNJ16	NM_018658.2	potassium voltage-gated channel subfamily J member 16	0.57	2.87E-02
UBD	NM_006398.3	ubiquitin D	0.58	4.04E-06
CYP3A7	NM_000765.4	cytochrome P450 family 3 subfamily A member 7	0.58	7.98E-04
TAGLN	NM_001001522.2	transgelin	0.58	3.98E-04
MIR133A1HG	NR_110369.1	MIR133A1 host gene	0.58	2.02E-09
HULC	NR_004855.2	hepatocellular carcinoma up-regulated long non-coding RNA	0.59	4.67E-03
F13B	NM_001994.2	coagulation factor XIII B chain	0.59	2.66E-02
IER3	NM_003897.3	immediate early response 3	0.59	9.75E-10

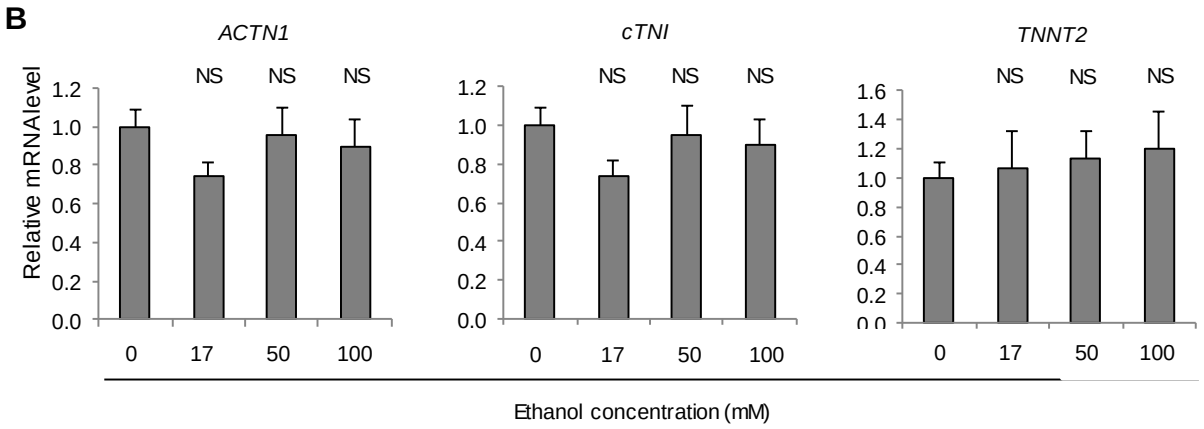


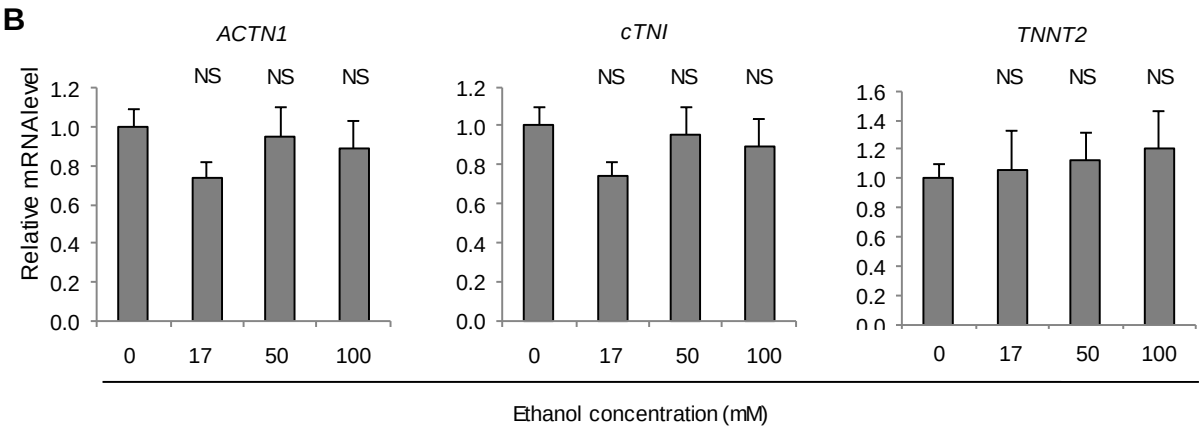
Supplementary Figure 1. Immunocytochemical analysis (ICC) of hiPSC-CMs revealing robust expression of CM-associated markers.



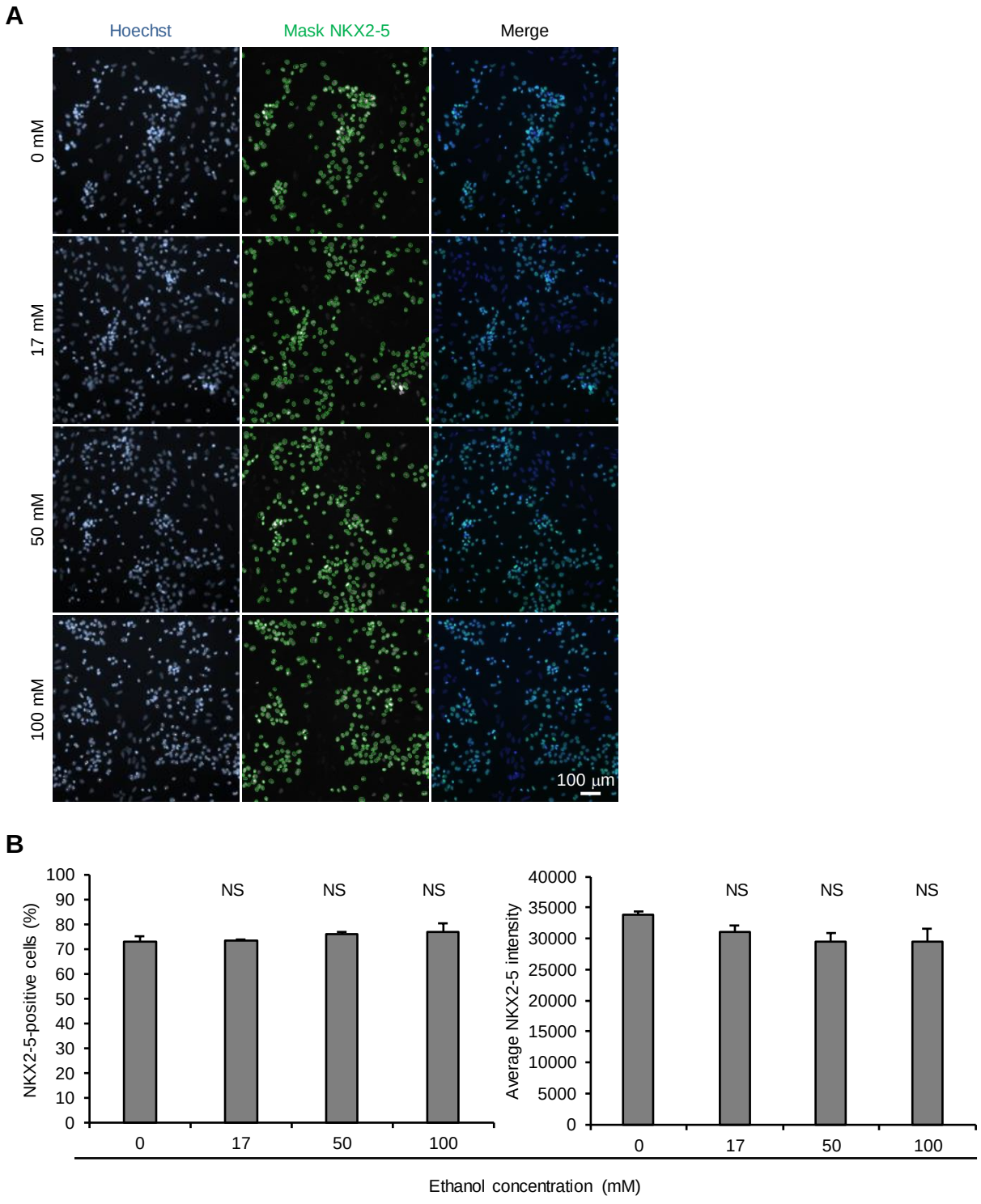
Supplementary Figure 2. Representative images of hiPSC-CMs for the detection of MitoSOX by ArrayScan related to Figure 3B. hiPSC-CMs were treated with ethanol for 5 days and stained with MitoSOX and Hoechst. Images were acquired and analyzed using ArrayScan. Mask, MitoSOX Red was quantified with a spot mask (ring-shape) that extended 7 units from the nucleus. Quantitative summary of average MitoSOX intensity and percentage of MitoSOX-positive cells are presented in Figure 3B.


A
0 mM
17 mM
50 mM
100 mM
α-actinin
NKX2-5
DAPI
100 μm


B
ACTN1
cTNI
TNNT2
NS
Relative mRNAlevel
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
0
17
50
100
Ethanol concentration (mM)

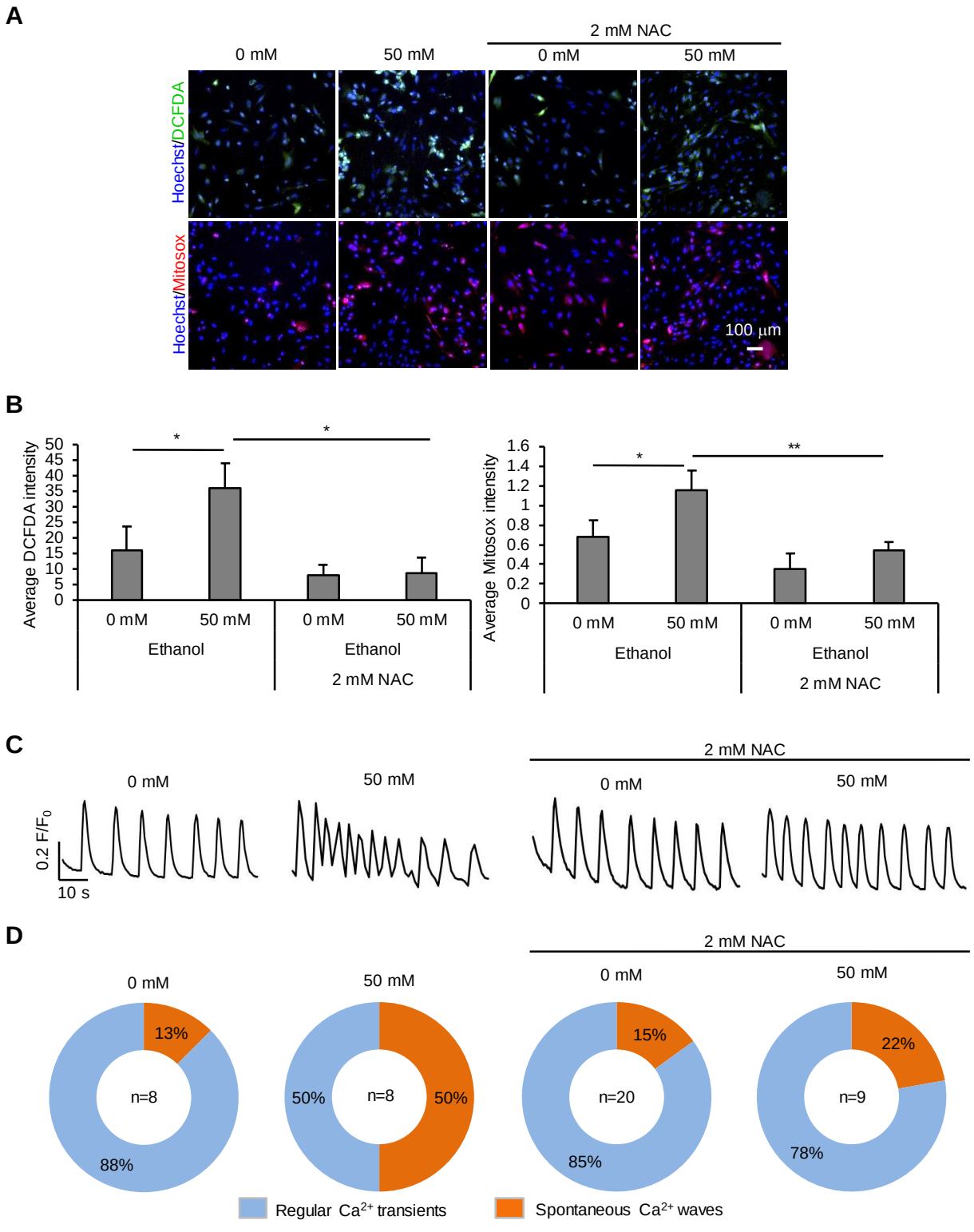

B
ACTN1
cTNI
TNNT2
NS
Relative mRNAlevel
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
0
17
50
100
Ethanol concentration (mM)

Supplementary Figure 3. Ethanol exposure of hiPSC-CMs has no effect on the expression of cardiac markers. (A) Immunocytochemical analysis showing the expression of cardiac transcription factor NKX2-5 and myocyte structural protein α-actinin. (B) qRT-PCR panel showing relative mRNA expression levels of cardiac markers including α-actinin, troponin I and troponin T. Data expressed as mean ± SD (n = 3).

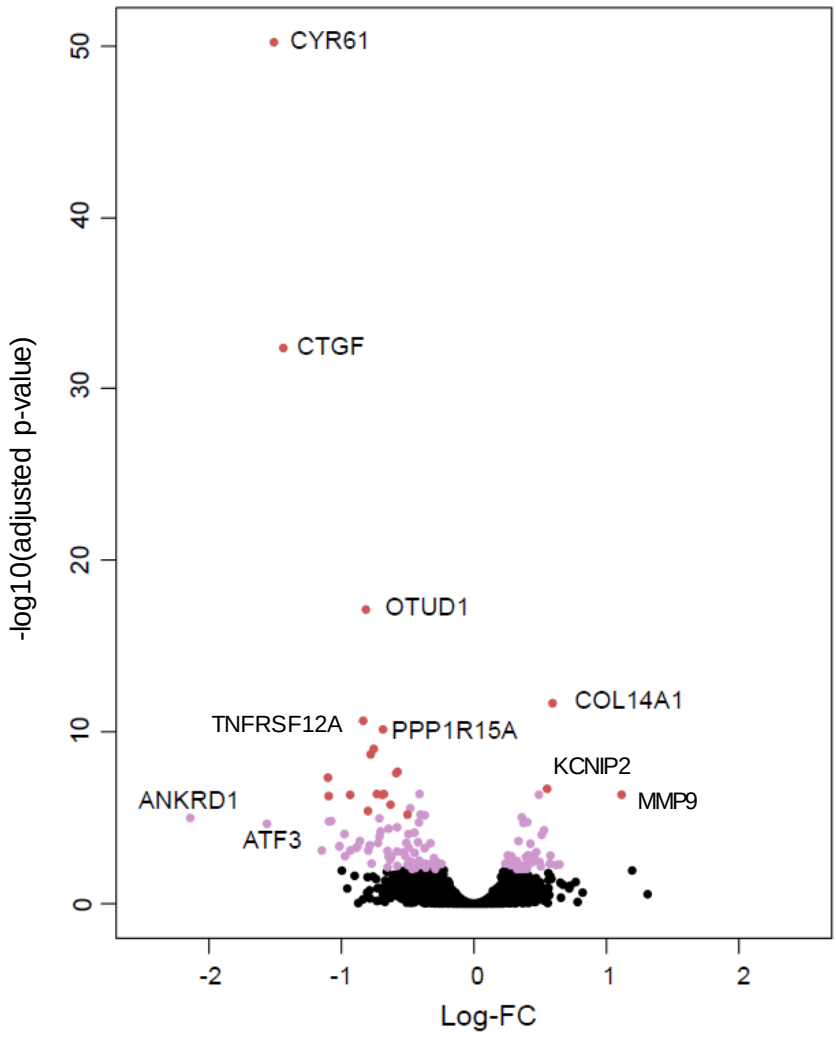


Supplementary Figure 4. Immunocytochemical analysis of hiPSC-CMs for the assessment of hiPSC-CM purity by ArrayScan related to Figure 4C. hiPSC-CMs were treated with ethanol for 5 days and stained with cardiac transcription factor NKX2-5 and

Hoechst. Images were acquired and analyzed using ArrayScan. NKX2-5 was quantified with mask modifier within a circular area restricted to the nuclear region. (A) Representative images. (B) Quantitative summary of percentage of NKX2-5-positive cells and average NKX2-5 intensity. Data presented as average fluorescence intensity per well and percentage of NKX2-5 positive cells, shown as mean ± SD (n = 5). NS = no significant difference compared with control (0 mM).


A
2 mM NAC
0 mM
50 mM
0 mM
50 mM
Hoechst/DCFDA
Hoechst/Mitosox
100 μm
B
Average DCFDA intensity
Average Mitosox intensity
0 mM
50 mM
Ethanol
2 mM NAC
C
0 mM
50 mM
2 mM NAC
0.2 F/F0
10 s
D
13%
88%
n=8
50%
50%
15%
85%
n=20
22%
78%
n=9
Regular Ca2+ transients
Spontaneous Ca2+ waves

Supplementary Figure 5. ROS scavenger N-acetyl cysteine (NAC) reduces the ethanol-induced ROS production and abnormal Ca^{2+} transients. hiPSC-CMs were pre-treated with ROS scavenger NAC at 2 mM for 1 h followed by the treatment with 0 or 50 mM ethanol in the presence of 2 mM NAC. hiPSC-CMs treated with 0 or 50 mM ethanol without NAC were used as controls. (A) Representative images of hiPSC-CMs for the detection of ROS by ArrayScan. hiPSC-CMs were stained with DCFDA and MitoSOX Red to detect intracellular (green) and mitochondrial ROS (red), respectively. Nuclei were stained with Hoechst (blue). (B) Analysis of ROS production through ArrayScan. Data presented as average fluorescence intensity per well for both DCFDA and MitoSOX Red, shown as mean ± SD (n = 4). *, p-value<0.05; **, p-value<0.01. (C) Representative fluorescent traces showing intercellular Ca^{2+} transients in cells. (D) Pie chart showing the percentage of cells exhibiting regular Ca^{2+} transients (blue) or Ca^{2+} transients with spontaneous Ca^{2+} waves (SCWs; orange). Sample sizes (n) are denoted in the center of the graphs for each groups.



Supplementary Figure 6. Volcano plot of hiPSC-CM transcriptome profiles. The transcriptome of ethanol-treated cells was compared with untreated cells. The x-axis specifies log2 transformed fold-changes (Log-FC) and the y-axis specifies –log10 transformed p-values (NLP) from DESeq2. Each spot represents a gene detected using RNA-seq. Black colored dots are genes below statistical significance threshold (p-value ≤ 0.05 after adjustment with Benjamini-Hochberg correction). Plum colored dots are genes with -log10(adjusted p-value) ≥ 2, and red colored dots are genes with -log10(adjusted p-value) ≥ 5. Text labeled genes are those with -log10(adjusted p-value) > 10 or Log-FC >1.5 or < -1.5.