Supplementary Information

Melphalan induces cardiotoxicity through oxidative stress in cardiomyocytes derived from human induced pluripotent stem cells

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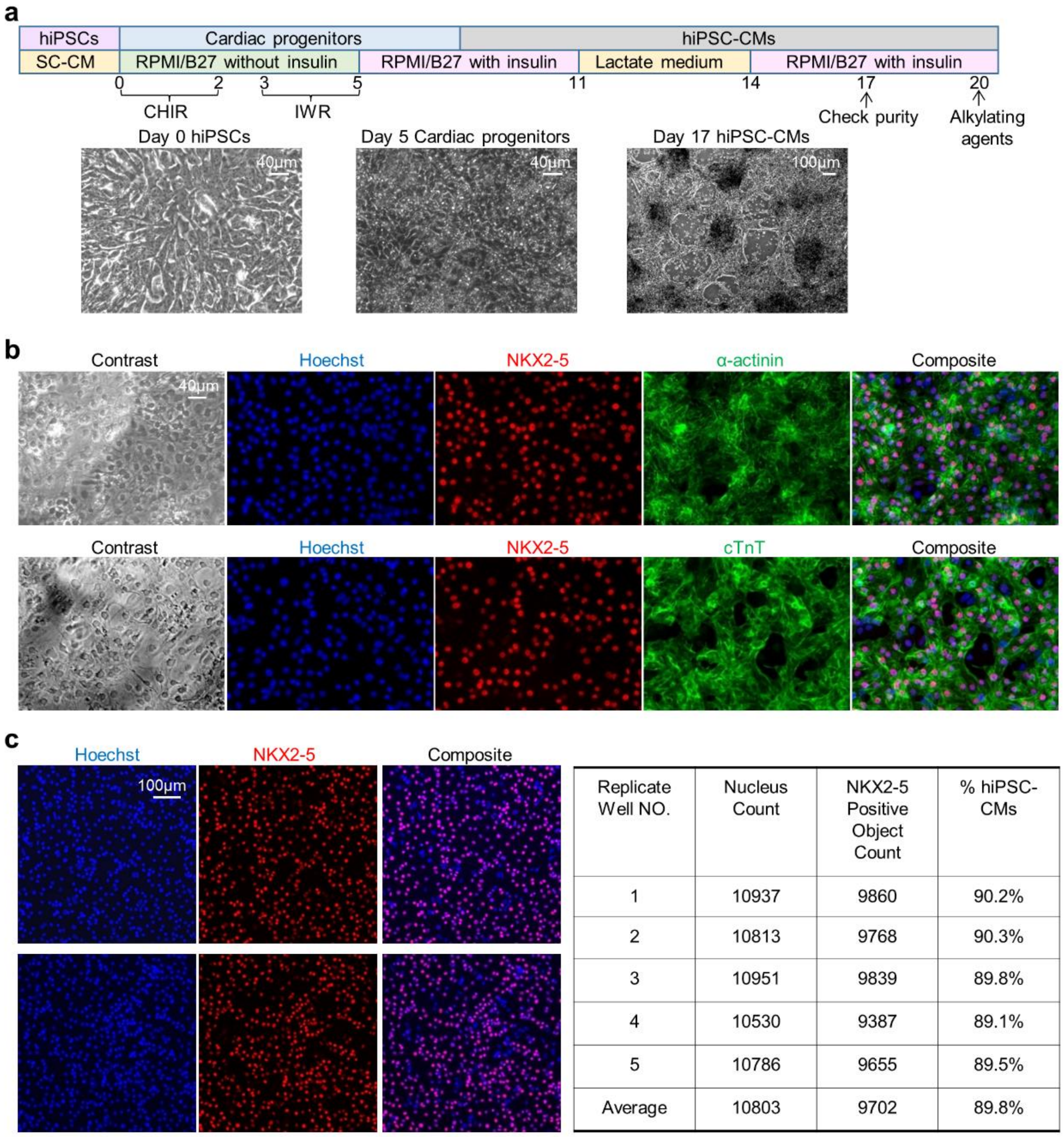

Replicate Well NO.	Nucleus Count	NKX2-5 Positive Object Count	% hiPSC-CMs
1	10937	9860	90.2%
2	10813	9768	90.3%
3	10951	9839	89.8%
4	10530	9387	89.1%
5	10786	9655	89.5%
Average	10803	9702	89.8%

Fig. S1 Directed differentiation of hiPSCs and generation of highly enriched hiPSC-CMs. a hiPSCs were induced for CM differentiation and hiPSC-CMs were enriched by metabolic selection. A parallel culture of hiPSC-CMs was harvested to determine CM purity at day 17, and the rest of the cells were cultured until day 20 for subsequent assessments. **b** Representative images of immunocytochemistry revealing the majority of the cells in culture were positive for cardiac transcription factor NKX2-5, and structural proteins cardiac troponin T and α-actinin at day 17. **c** Representative images acquired from ArrayScan and quantitative summary of percentage of NKX2-5-positive cells (~90%) indicated highly enriched CMs were generated in the cultures at day 17. SC-CM, stem cell culture medium.

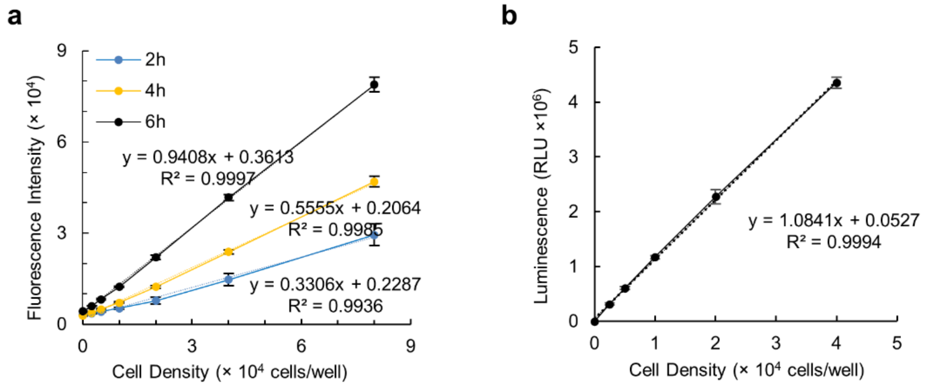

Fig. S2 Validation of CellTiter-Blue and CellTiter-Glo 3D Cell Viability Assays. hiPSC-CMs were seeded at a range of numbers from 0.25 to 4 or 8 × 10^4 cells per well, cultured for 24 h, and then incubated the cells with CellTiter-Blue reagent for 2, 4, and 6 h or CellTiter-Glo 3D reagent, respectively. **a** Representative plot presenting the linear relationship between hiPSC-CM numbers and fluorescence intensity via CellTiter-Blue Viability Assay (n = 5). **b** Representative plot presenting the linear relationship between hiPSC-CM numbers and luminescence intensity of via CellTiter-Glo 3D Viability Assay (n = 5). Note: The readout of fluorescence or luminescence intensity had a nearly linear relationship with the number of cells ($R^2 > 0.99$), suggesting that these two assays were reliable and sensitive for the estimation of cell numbers of hiPSC-CMs.

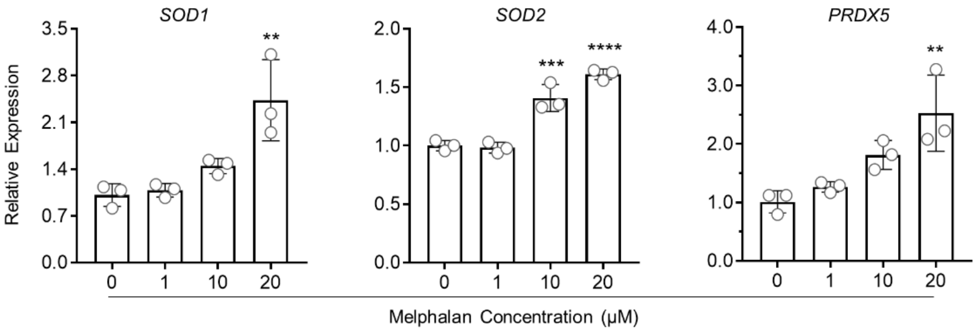

Fig. S3 Melphalan treatment of hiPSC-CMs induces oxidative stress. qRT-PCR analysis showing relative gene expression levels of oxidative stress-related genes including *SOD1*, *SOD2*, and *PRDX5* in hiPSC-CMs treated with melphalan for 3 days (n = 3). Comparisons were conducted between each treatment group and no melphalan group via One-way ANOVA test. **, *P*-value < 0.01; ***, *P*-value < 0.001; ****, *P*-value < 0.0001.

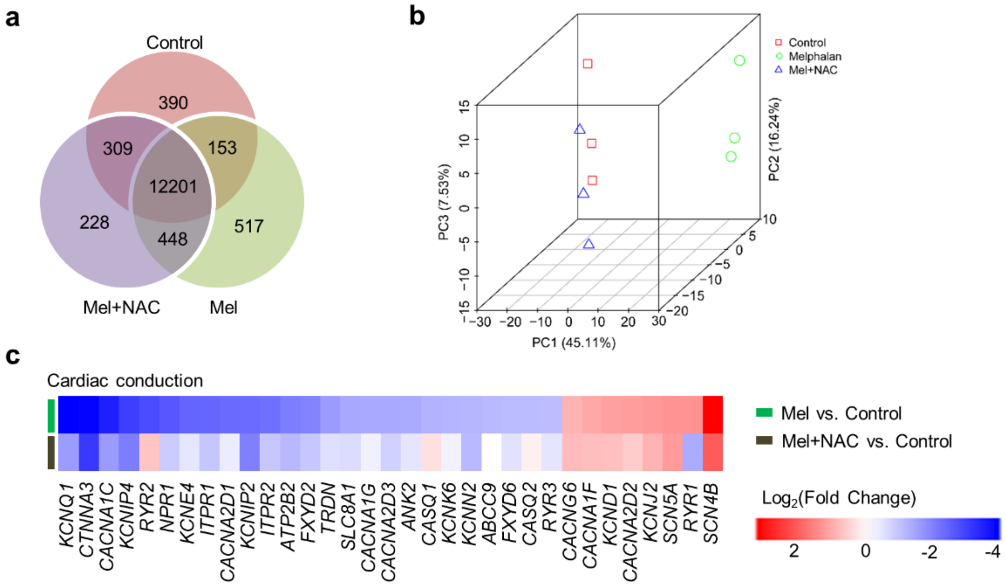

Fig. S4 NAC attenuates melphalan-induced alteration of hiPSC-CM transcriptome profiles characterized by RNA-Seq analysis. RNA-Seq analysis of hiPSC-CMs upon 0 and 20 µM of melphalan treatment with or without NAC supplementation for 3 days (n = 3). **a** Venn diagram showing the amounts of commonly and uniquely expressed genes among different treatment groups. **b** Principal component analysis plot showing the differences between groups and the distribution of samples in each group. **c** Heatmap showing the DEGs involved in GO term of cardiac conduction in melphalan- or Mel+NAC-treated hiPSC-CMs compared with control group, respectively. Blue and red colors of displayed rectangles indicate the levels of gene expression according to $\log_2$(fold change). Control, no melphalan; Mel, 20 µM melphalan; Mel+NAC, 20 µM melphalan with 1 mM NAC.

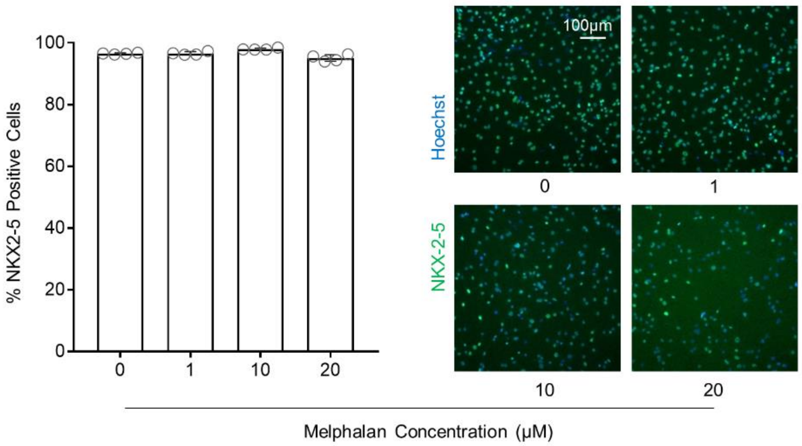

Fig. S5 Melphalan treatment does not alter hiPSC-CM purity. hiPSC-CM purity was determined via ArrayScan. Cells were fixed and stained with first and secondary antibodies to detect NKX2-5. Nuclei were stained with Hoechst upon melphalan treatment for 5 days. NKX2-5-positive cells emitted bright green nuclear fluorescence. Representative images and quantification of percentage of NKX2-5-positive cells were shown (n = 4). Comparisons were conducted between each treatment group and no melphalan group via One-way ANOVA test.

Table S1. Information of major reagents

Product Name	Supplier	Catalog#
mTeSR1 defined medium	Stem Cell Technologies	85850
Versene	Thermo Fisher Scientific	15040066
RPMI 1640 medium	Thermo Fisher Scientific	11875093
B27 Supplement (50×), minus insulin	Thermo Fisher Scientific	A1895601
B27 Supplement (50×), serum free	Thermo Fisher Scientific	17504044
RPMI1640, no glucose	Thermo Fisher Scientific	11879020
Sodium DL-lactate solution	Sigma	L4263
Matrigel	Thermo Fisher Scientific	CB40230C
CHIR99021	Selleckchem	S2924
IWR1	Sigma	I0161
paraformaldehyde	Electron Microscopy Sciences	15710
Hoechst33342	Thermo Fisher Scientific	H3570
melphalan	Selleckchem	S8266
dimethyl sulfoxide	Sigma	D2438
N-Acetyl-L-cysteine	Sigma	A9165
fetal bovine serum	GE Healthcare Life Sciences, Hyclone Laboratories	SH30396.03
Live Cell Imaging Solution	Thermo Fisher Scientific	A14291DJ
CellTiter-Blue Cell Viability Assay	Promega	G8081
CellTiter-Glo 3D Cell Viability Assay	Promega	G9683
CellEvent Caspase-3/7 Green Detection Reagent	Thermo Fisher Scientific	C10423
Image-iT LIVE Green Reactive Oxygen Species Detection Kit (DCFDA)	Thermo Fisher Scientific	I36007
MitoSOX Red Mitochondrial Superoxide Indicator	Thermo Fisher Scientific	M36008
Fluo-4, AM	Thermo Fisher Scientific	F14201
Aurum total RNA mini kit	Bio-Rad	732-6820
SuperScript VILO cDNA Synthesis Kit	Thermo Fisher Scientific	11754050
iTaq SyBr green master mix	Bio-Rad	172-5121

Table S2. Antibodies for immunocytochemistry

Type	Target	Isotype	Supplier	Catalog#	Dilution
Primary	α-actinin	mouse IgG$_1$	Sigma	A7811	1:800
	NKX2-5	rabbit IgG	Cell Signaling Technologies	SC14033	1:1600
	cardiac troponin T	mouse IgG$_1$	Fisher Scientific	MS295P1	1:200
Secondary	Alexa 488, Goat anti-mouse IgG1		Invitrogen	A-21121	1:1000
	Alexa 594, Goat anti-rabbit IgG		Invitrogen	A-11012	1:1000
	Alexa 488, Goat anti-rabbit IgG		Invitrogen	A-11034	1:1000

Table S3. SyBr green primers for qRT-PCR

Gene	Full name	Accession code	Primer
BCL2	B-cell CLL/lymphoma 2	NM_000633.2	Forward: GAGAAATCAAACAGAGGCCG Reverse: CTGAGTACCTGAACCGGCA
BAX	BCL2-associated X protein	NM_004324	Forward: GGAGGAAGTCCAATGTCCAG Reverse: TCTGACGGCAACTTCAACTG
SOD1	Superoxide dismutase 1	NM_000454	Forward: GGTGGGCCAAAGGATGAAGAG Reverse: CCACAAGCCAAACGACTTCC
SOD2	Superoxide dismutase 2	NM_000636	Forward: GCTCCGGTTTTGGGGTATCTG Reverse: GCGTTGATGTGAGGTTCCAG
SOD3	Superoxide dismutase 3	NM_003102	Forward: ATGCTGGCGCTACTGTGTTC Reverse: CTCCGCCGAGTCAGAGTTG
GSR	Glutathione reductase	NM_001195102	Forward: CACTTGCGTGAATGTTGGATG Reverse: TGGGATCACTCGTGAAGGCT
NQO2	NAD(P)H dehydrogenase, quinone 2	NM_000904	Forward: GTACTCATTGTCTATGCACACCA Reverse: TGCCTGCTCAGTTCATCTACA
GPX1	Glutathione peroxidase 1	NM_201397	Forward: CAGTCGGTGTATGCCTTCTCG Reverse: GAGGGACGCCACATTCTCG
PRDX5	Peroxiredoxin 5	NM_181651	Forward: TCCTGGCTGATCCCACTGG

			Reverse: CTGTGAGATGATATTGGGTGCC
RYR2	Ryanodine receptor 2	NM_001035	Forward: CAAATCCTTCTGCTGCCAAG Reverse: CGAAGACGAGATCCAGTTCC
CACNA1C	Calcium channel, voltage-dependent, L type, alpha 1C subunit	NM_000719	Forward: TTTTAAAAACGCTTCCACCG Reverse: TTCCAGAAGATGATTCCAACG
TNNI1	Troponin I type 1	NM_003281	Forward: AGCATCAGGCTCTTCAGCA Reverse: ACAGTCTGCAGTCTACGGCG
TNNT2	Troponin T type 2	NM_001001431	Forward: GCGGGTCTTGGAGACTTTCT Reverse: TTCGACCTGCAGGAGAAGTT
MYH6	Myosin heavy chain 6	NM_002471	Forward: CTTCTCCACCTTAGCCCTGG Reverse: GCTGGCCCTTCAACTACAGA
MYH7	Myosin heavy chain 7	NM_000257	Forward: CGCACCTTCTTCTCTTGCTC Reverse: GAGGACAAGGTCAACACCCT
MYL2	Myosin light chain 2	NM_000432	Forward: CGTTCTTGTCAATGAAGCCA Reverse: CAACGTGTTCTCCATGTTCG
MYL7	Myosin light chain 7	NM_021223	Forward: CTTGTAGTCGATGTTCCCCG Reverse: TCAAGCAGCTTCTCCTGACC

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_001256799	Forward: CTGGGCTACACTGAGCACC Reverse: AAGTGGTCGTTGAGGGCAATG

Table S4. List of top 20 DEGs and enriched GO terms in hiPSC-CMs treated with melphalan compared with no melphalan treatment based on proteomic analysis.

Gene Symbol	Gene Description	Fold Change	-Log$_{10}$(*P*-value)
Up-regulated			
TNFRSF10C	Tumor necrosis factor receptor superfamily member 10c	3.900	3.14286
HBD	Hemoglobin Subunit Delta	2.974	1.88666
DDB2	Damage Specific DNA Binding Protein 2	2.963	2.81423
HBA1	Hemoglobin Subunit Alpha 1	2.445	1.81212
RRM2	Ribonucleotide Reductase Regulatory Subunit M2	2.391	2.16014
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21 Cip1)	2.383	1.65025
NFE2L2	Nuclear Factor, Erythroid 2 Like 2	2.271	1.82614
AMOT	Angiomotin	2.194	2.00372
APOA1	Apolipoprotein A1	2.193	1.84905
S100A13	S100 Calcium Binding Protein A13	2.178	2.20418
Down-regulated			
MRPS18A	Mitochondrial Ribosomal Protein S18A	0.601	4.26649
RPL32	Ribosomal Protein L32	0.424	4.03046
TRIM24	Tripartite Motif Containing 24	0.661	4.00693
HNRNPD	Heterogeneous Nuclear Ribonucleoprotein D	0.385	3.79572
PNN	Pinin, Desmosome Associated Protein	0.653	3.56729
TBX20	T-Box Transcription Factor 20	0.419	3.39066
PRKCA	Protein Kinase C Alpha	0.572	3.24993
STXBP6	Syntaxin Binding Protein 6	0.638	3.09285
RPL19	Ribosomal Protein L19	0.422	3.07857
RPL8	Ribosomal Protein L8	0.457	3.06067
GO Term ID	GO Term Description	Gene Count	*P*-value
Up-regulated			
GO:0051346	Negative regulation of hydrolase activity	16	1.20842E-11
GO:0043086	Negative regulation of catalytic activity	20	8.6046E-11
GO:0050819	Negative regulation of coagulation	8	5.81985E-10
GO:0042060	Wound healing	16	8.1444E-10

GO:0061041	Regulation of wound healing	10	9.43747E-10
GO:0009611	Response to wounding	17	1.07669E-09
GO:0044092	Negative regulation of molecular function	21	1.16815E-09
GO:0050818	Regulation of coagulation	9	1.20956E-09
GO:1903034	Regulation of response to wounding	10	3.22653E-09
GO:0007596	Blood coagulation	13	3.64145E-09
Down-regulated			
GO:0003735	Structural constituent of ribosome	50	3.63833E-53
GO:0005198	Structural molecule activity	58	2.19188E-34
GO:0044822	Poly(A) RNA binding	67	6.16268E-33
GO:0003723	RNA binding	73	1.16359E-29
GO:0003676	Nucleic acid binding	97	2.70215E-20
GO:1901363	Heterocyclic compound binding	107	1.09761E-13
GO:0097159	Organic cyclic compound binding	107	2.96819E-13
GO:0019843	rRNA binding	11	4.7287E-10
GO:0050839	Cell adhesion molecule binding	19	7.47353E-07
GO:0098641	Cadherin binding involved in cell-cell adhesion	15	1.16713E-06

Table S5. List of top 20 DEGs, enriched GO terms and KEGG pathways in hiPSC-CMs treated with melphalan compared with no melphalan treatment based on RNA-Seq analysis.

Gene Symbol	Gene Description	Log_2(Fold Change)	Adjusted *P*-value
Up-regulated			
GDF15	Growth differentiation factor 15	4.4469	3.69E-268
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21 Cip1)	4.224	0
DUSP13	Dual specificity phosphatase 13	3.9975	1.01E-33
GRHL3	Grainyhead-like transcription factor 3	4.3444	7.80E-27
DRAXIN	Dorsal inhibitory axon guidance protein	3.948	6.61E-33
ZSCAN4	Zinc finger and SCAN domain containing 4	3.8087	1.33E-19
FDXR	Ferredoxin reductase	3.798	0
TNFRSF10C	Tumor necrosis factor receptor superfamily member 10c	3.729	1.65E-138
SCN4B	Sodium channel voltage gated type IV beta subunit	3.6045	2.96E-29
BPIFA1	BPI fold containing family A member 1	3.5181	3.98E-14
Down-regulated			
GPC6	Glypican 6	-6.3842	0
CNTN1	Contactin 1	-6.0072	3.36E-74
SDK1	Sidekick cell adhesion molecule 1	-5.7863	4.10E-81
CDH13	Cadherin 13	-5.5657	1.47E-101
THSD4	Thrombospondin type 1 domain containing 4	-5.5175	1.50E-199
PARK2	Parkin RBR E3 ubiquitin protein ligase	-5.4659	4.31E-48
SLC24A3	Solute carrier family 24 member 3	-5.4454	6.87E-50
MSRA	Methionine sulfoxide reductase A	-5.2313	1.21E-63
PARD3B	Par-3 family cell polarity regulator beta	-5.2087	5.45E-75
CTNND2	Catenin delta 2	-5.0364	1.20E-108
GO Term ID	GO Term Description	Gene Count	Adjusted *P*-value
Down-regulated			
GO:0030198	extracellular matrix organization	93	2.62E-23
GO:0043062	extracellular structure organization	96	8.13E-21
GO:0006936	muscle contraction	78	3.10E-15

GO:0003012	muscle system process	83	5.42E-12
GO:0007416	synapse assembly	47	7.21E-12
GO:0060047	heart contraction	58	3.44E-10
GO:0003015	heart process	59	3.44E-10
GO:0050808	synapse organization	74	3.44E-10
GO:0030199	collagen fibril organization	22	9.01E-10
GO:0051965	positive regulation of synapse assembly	25	1.69E-09
GO:0061448	connective tissue development	55	2.67E-09
GO:0060537	muscle tissue development	70	4.90E-09
GO:1903522	regulation of blood circulation	57	4.90E-09
GO:1904018	positive regulation of vasculature development	45	4.90E-09
GO:0051963	regulation of synapse assembly	31	7.11E-09
GO:0051216	cartilage development	45	1.28E-08
GO:0034765	regulation of ion transmembrane transport	77	1.56E-08
GO:0050804	modulation of chemical synaptic transmission	73	1.59E-08
GO:0099177	regulation of trans-synaptic signaling	73	1.68E-08
GO:0031589	cell-substrate adhesion	63	2.18E-08
KEGG Pathway ID	KEGG Pathway Description	Gene Count	Adjusted *P*-value
hsa04512	ECM-receptor interaction	28	5.26E-06
hsa04020	Calcium signaling pathway	47	5.26E-06
hsa05414	Dilated cardiomyopathy (DCM)	29	5.82E-06
hsa04115	p53 signaling pathway	25	7.86E-06
hsa05410	Hypertrophic cardiomyopathy (HCM)	27	7.86E-06
hsa04261	Adrenergic signaling in cardiomyocytes	36	7.28E-05
hsa04974	Protein digestion and absorption	25	0.000136
hsa04510	Focal adhesion	44	0.000145
hsa04270	Vascular smooth muscle contraction	32	0.00023
hsa05412	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	22	0.00023
hsa04911	Insulin secretion	24	0.000236
hsa04010	MAPK signaling pathway	56	0.000269
hsa04080	Neuroactive ligand-receptor interaction	57	0.000269

hsa04151	PI3K-Akt signaling pathway	61	0.000269
hsa04933	AGE-RAGE signaling pathway in diabetic complications	26	0.000269
hsa04514	Cell adhesion molecules (CAMs)	30	0.000269
hsa04360	Axon guidance	40	0.000277
hsa04925	Aldosterone synthesis and secretion	25	0.000381
hsa04670	Leukocyte transendothelial migration	27	0.000495
hsa04015	Rap1 signaling pathway	42	0.00054

Table S6. List of top 20 DEGs, enriched GO terms and KEGG pathways in melphalan-treated hiPSC-CMs with NAC supplementation compared with no supplementation based on RNA-Seq analysis.

Gene Symbol	Gene Description	Log_2(Fold Change)	Adjusted *P*-value
Up-regulated			
GPC6	Glypican 6	3.5211	7.20E-97
THSD4	Thrombospondin type 1 domain containing 4	3.3196	1.51E-65
SMOC2	SPARC related modular calcium binding 2	3.1634	3.82E-34
SPOCK1	Sparc/osteonectin cwcv and kazal-like domains proteoglycan (testican) 1	3.0824	1.03E-214
LEFTY2	Left-right determination factor 2	3.0782	1.03E-223
COL21A1	Collagen type XXI alpha 1	2.9993	3.31E-68
FGF1	Fibroblast growth factor 1 (acidic)	2.9685	1.46E-48
SORCS3	Sortilin-related VPS10 domain containing receptor 3	2.9096	2.93E-55
ACTA1	Actin alpha 1 skeletal muscle	2.8581	1.07E-201
LMOD2	Leiomodin 2 (cardiac)	2.8378	4.30E-205
Down-regulated			
ZSCAN4	Zinc finger and SCAN domain containing 4	-2.1307	1.51E-14
SCN2A	Sodium channel voltage gated type II alpha subunit	-2.0074	2.82E-28
GRIN2C	Glutamate receptor ionotropic N-methyl D-aspartate 2C	-1.6951	1.09E-10
CEND1	Cell cycle exit and neuronal differentiation 1	-1.5414	2.87E-40
DRAXIN	Dorsal inhibitory axon guidance protein	-1.5412	5.01E-13
VGF	VGF nerve growth factor inducible	-1.5388	4.13E-08
TRIM49C	Tripartite motif containing 49C	-1.5274	1.28E-07
TAF11L11	TATA-Box Binding Protein Associated Factor 11 Like 11	-1.5038	2.90E-07
P2RX6	Purinergic receptor P2X ligand gated ion channel 6	-1.4743	2.58E-13
KLLN	Killin, p53-regulated DNA replication inhibitor	-1.4282	9.33E-29
GO Term ID	GO Term Description	Gene Count	Adjusted *P*-value
Up-regulated			
GO:0030198	extracellular matrix organization	66	1.10E-29
GO:0043062	extracellular structure organization	68	4.73E-28
GO:0030199	collagen fibril organization	18	2.72E-12

GO:0006936	muscle contraction	43	4.60E-12
GO:0007517	muscle organ development	44	5.82E-11
GO:0031589	cell-substrate adhesion	41	7.67E-11
GO:0003012	muscle system process	46	9.16E-11
GO:0010810	regulation of cell-substrate adhesion	30	6.84E-10
GO:0003015	heart process	33	1.23E-08
GO:0030239	myofibril assembly	17	1.29E-08
GO:1903522	regulation of blood circulation	33	1.79E-08
GO:0060047	heart contraction	32	1.93E-08
GO:0008016	regulation of heart contraction	29	9.10E-08
GO:0061448	connective tissue development	30	2.25E-07
GO:0055002	striated muscle cell development	23	2.39E-07
GO:0010927	cellular component assembly involved in morphogenesis	19	2.69E-07
GO:0060537	muscle tissue development	36	8.84E-07
GO:0055001	muscle cell development	23	9.21E-07
GO:0070252	actin-mediated cell contraction	18	2.04E-06
GO:0090130	tissue migration	30	2.06E-06
KEGG Pathway ID	KEGG Pathway Description	Gene Count	Adjusted *P*-value
hsa04933	AGE-RAGE signaling pathway in diabetic complications	20	2.01E-08
hsa04020	Calcium signaling pathway	27	2.07E-08
hsa04974	Protein digestion and absorption	18	2.46E-08
hsa04512	ECM-receptor interaction	16	9.40E-07
hsa04270	Vascular smooth muscle contraction	19	3.22E-06
hsa04510	Focal adhesion	24	3.22E-06
hsa04925	Aldosterone synthesis and secretion	15	2.95E-05
hsa05410	Hypertrophic cardiomyopathy (HCM)	14	2.95E-05
hsa04066	HIF-1 signaling pathway	14	0.000107
hsa04151	PI3K-Akt signaling pathway	28	0.000141
hsa04921	Oxytocin signaling pathway	18	0.000141
hsa04022	cGMP-PKG signaling pathway	18	0.000232
hsa05414	Dilated cardiomyopathy (DCM)	13	0.000232
hsa04911	Insulin secretion	12	0.000519

hsa05146	Amoebiasis	12	0.000782
hsa04010	MAPK signaling pathway	24	0.000782
hsa04350	TGF-beta signaling pathway	12	0.001084
hsa05205	Proteoglycans in cancer	18	0.001737
hsa04924	Renin secretion	10	0.001737
hsa05412	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	10	0.002371