Supplementary methods

Time-lapse videos

Adult Cardiomyocytes isolated from 8-12 weeks old rats and plated in 24 well-plates, transfected with either cel-miR-67 or miR-1825 were imaged for 20 hours with an image every 15 minutes. EVOS FL Auto Cell Imaging System (ThermoFisher Scientific) with an EVOS Onstage Incubator was used to keep cells in a regulated environment (37°C, 5% CO_2, Humidity). Bright field images were acquired at 20 × magnification.

Isolation of adult rat ventricular cardiomyocytes

Hearts were extracted from anesthetized rats and perfused using Krebs-Hanseleit bicarbonate (KHB) buffer. At time 10 min, 12.5 µl of 0.1 $CaCl_2$ was added to the circulation buffer solution. At time 15 min, 25 µl of 0.1 M $CaCl_2$ was added to make final concentration of 0.1 mM. Heart was then minced in KHB and filtered using a nylon mesh. Cells were then spun at ~300 rpm for 3 min at RT. Next, cells were re-suspended in solution containing 10% bovine serum albumin (BSA; sigma) in 1:1 ratio with KHB. 0.1 M $CaCl_2$ was added at 5 min intervals (3 times; 50 µl, 75 µl, and 100 µl) to achieve a final concentration of 1 mM [1].

Cells were then separated by sedimentation and re-suspended in DMEM containing 5% FBS and 1% penicillin-streptomycin (P/S). Pre-plating the cells in non-laminin coated plates was used to remove fibroblast contamination. Finally, cells were plated on mouse-laminin (Life Technologies, CA) coated plates and cultured at 37°C in 5% CO_2 and humidified atmosphere.

Transfection efficiency

Adult Cardiomyocytes were transfected with fluorescent labelled miRNA Dy547-cel-miR-67 using standard transfection protocol. Cells were imaged each day post transfection to check for RFP positive cells. Cytotoxic siRNA against Ubiquitin-C (UbC) siUbC was transfected using standard protocol and cells were fixed 4 days post transfection and stained for Troponin-I (Cardiomyocyte) and DAPI (nucleus). Control cells were transfected with cel-miR-67.

Immuno-staining

Cells were fixed with paraformaldehyde (4%) and incubated with primary antibodies overnight. Primary antibodies used included: rabbit mono-clonal antibody against Troponin-I (Santa Cruz Biotechnology sc-15368), histone H3 phosphorylation at serine 10 (Cell Signaling 9706S), Aurora B kinase (Abcam ab3609), VDAC (Cell Signaling 4661P), Nkx2.5 (Santa Cruz H114), and Anti-Oxoguanine 8 antibody (Abcam ab64548). Cells were then washed with PBS and incubated for 1 hour at RT with secondary antibodies conjugated to Alexa Fluor-488, -555, or -647 (Life Technologies). Click-IT EdU 555 and 647 imaging kit was used to identify EdU incorporation according to the manufacturer's instructions. DAPI was used to stain nuclei. Dihydrorhodamine 123 (Life Technologies, D-23806) was used to detect ROS by reconstituting in DMSO. Cells were incubated in 10 uM dihydrorhodamine 123 for 20 mins in the dark, followed by PBS wash. Wheat Germ Agglutinin (WGA), Alexa Fluor 488 conjugate (Invitrogen) was used to mark cell wall, as per manufacturer's protocol (Life Technologies, CA).

CM proliferation was measured by counting EdU (/pH3/ArB)$^+$ TnI$^+$ DAPI$^+$ CM per image. More than 3 images per condition, per trial (>1000 cells) were counted blindly by at least two individuals.

Cardiomyocyte cell count

Adult rat cardiomyocytes were transfected with either control cel-miR-67 or miR-1825 and each day post transfection a set of control and miR-1825 were fixed with 4% PFA, up until day 7. Fixed Cells were then permeabilized and stained with Tropinin-I (cardiac marker). Cardiomyocytes were counted for each set and fold change was calculated by normalizing the number of cells to its corresponding control (cel-miR-67 transfected) cells ($n \geq 3$).

Luciferase assay

3'UTR sequences used were as follows:

NDUFA10: >HmiT088389 3'UTR (binding sites in bold)

acgggccgccttctgctccagctgcatcacagtgatggccaagctgcatcagccgcactctcctggacgccatatagctttaagatc-
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NDUFA10-mut: CS-HmiT088389-MT05-01 (with all 4 CACTGG sites deleted indicated above in bold).

Rb1: >HmiT016131-MT05 3'UTR (binding site in bold)

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Meis2: >HmiT011163-MT05 3'UTR (binding site in bold)

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Immuno-histochemsity

Animals were euthanized with an overdose of sodium pentobarbital and hearts were harvested on day 30 post surgery. Hearts were fixed in 4% paraformaldehyde (PFA) for 24 hours, followed by paraffin sectioning. Masson's trichrome and Hematoxylin and eosin (H&E) staining was performed. EdU and WGA staining were performed according to the manufacturer's instructions.

Human induced-pluripotent stem cell derived cardiomyocytes (HiPS)

HiPS-CMs were commercially purchased from Cellular Dynamics International (Wisconsin, USA) and cultured according to manufacturer's protocol. Cells were transfected according to the previously mentioned protocol and harvested on day 5 post transfection.

Western blot

Primary antibodies used: P16 (Cell Signaling ab51243), CyclinD1 (Cell Signaling 2922S), β-actin (Cell Signaling 4970S), Bcl-2 (Cell Signaling 2870S), NDUFA10 (Santa Cruz sc-107807), phospho-Rb (Cell Signaling 9308S), Meis1/2 (Santa Cruz sc-10599), COX-IV (Cell Signaling 4850).

RNA-sequencing analysis

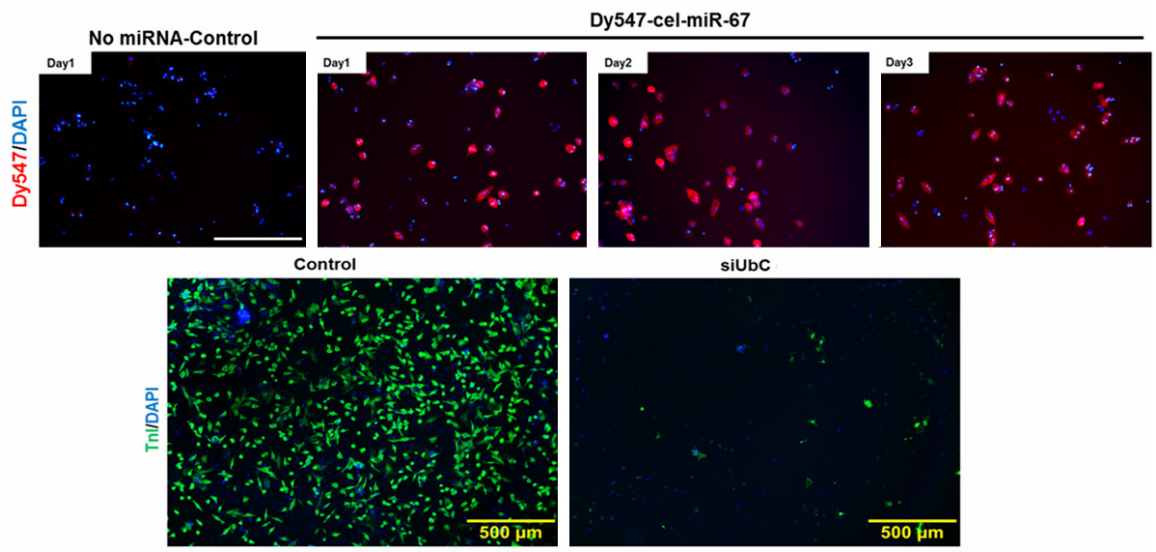
Adult CMs transfected with cel-miR-67 or hsa-miR-1825 were used to isolate total RNA using Trizol (Invitrogen) according to the manufacturer's protocol. RNA was treated with DNase to remove contaminating DNA and cleaned with RNeasy kits (Qiagen). Sequencing was performed at Cincinnati Children's Hospital's DNA sequencing and Genotyping core using Illumina HiSeq2500. Single end 50 bp reads with 10 million reads were generated for each sample. Each library was mapped directly to the rat genome (version 6.0) allowing two mismatches per read and a 50% overlap with CLC Genomics (Qiagen). Each read was permitted to be mapped to 10 locations. Expression values were based on the number of unique reads mapped to each gene based upon the total number of reads mapped to the entire genome (TPM). Two different statistical tests (Kal's and Baggerly's Tests) were utilized which followed a false detection rate of $P<0.05$. Enrichment for specific gene ontology categories were examined using DAVID [2], g: Profiler [3], BiNGO [4], and PANTHER [5].

Primer sequences

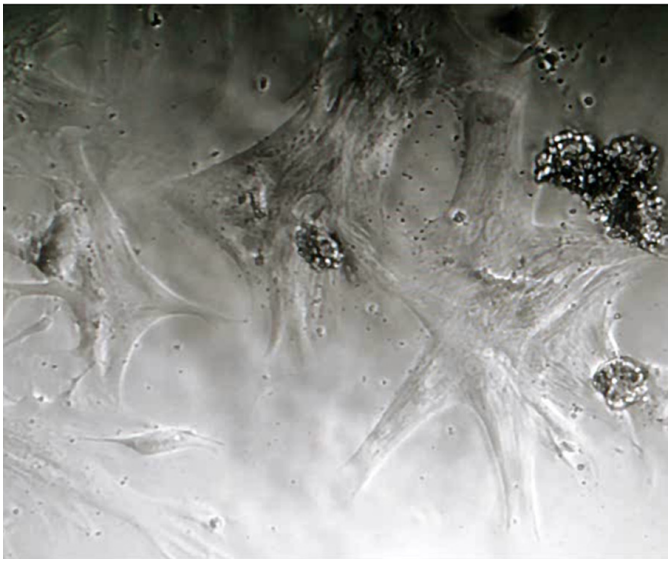
NDUFA10:
F: TGAATTACACGACCGTCCCG
R: TCCACTTGTCACCCACATCG
COX-1:
F: ATCGCAATTCCTACAGGCGT
R: TGTTAGGCCCCCTACTGTGA
B-actin:
F: ACCCTAAGGCCAACCGTGAAA
R: GTACGACCAGAGGCATACAGG
GAPDH:
F: GCCAAAAGGGTCATCATCTCCG
R: ACATTGGGGGTAGGAACACGGA
MYH6:
F: TGATGACTCCGAGGAGCTTT
R: TGACACAGACCCTTGAGCAG
MYH7:
F: CCTCGCAATATCAAGGGAAA
R: CTACAGGTGCATCAGCTCCA
NKX2.5
F: ACCGCCCCTACATTTTATCC
R: GACAGGTACCGCTGTTGCTT

References

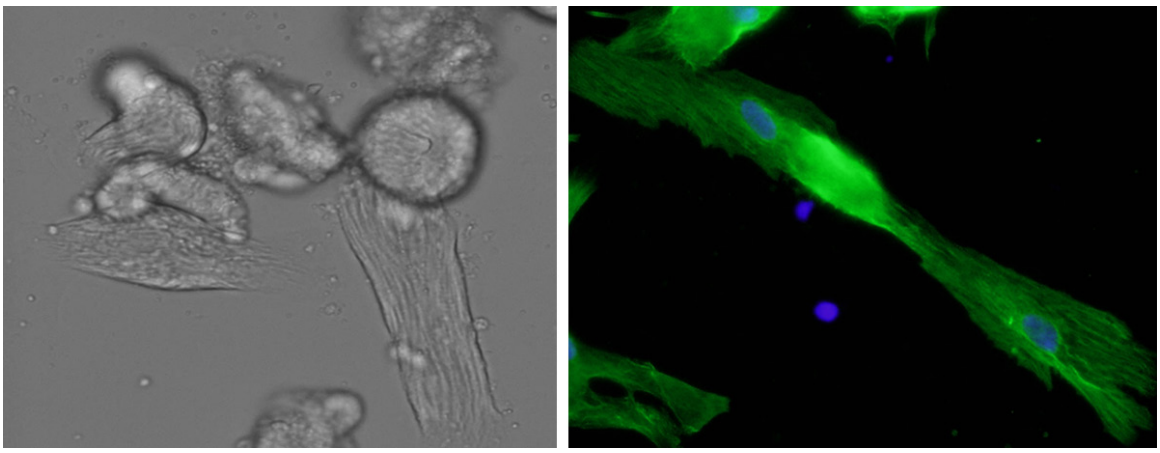
[1] Bell RM, Mocanu MM, Yellon DM. Retrograde heart perfusion: the langendorff technique of isolated heart perfusion. J Mol Cell Cardiol 2011; 50: 940-950.
[2] Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009; 4: 44-57.
[3] Reimand J, Arak T, Vilo J. G: profiler--a web server for functional interpretation of gene lists (2011 update). Nucleic Acids Res 2011; 39: W307-15.
[4] Maere S, Heymans K, Kuiper M. BiNGO: a cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. Bioinformatics 2005; 21: 3448-3449.
[5] Mi H, Muruganujan A, Casagrande JT, Thomas PD. Large-scale gene function analysis with the PANTHER classification system. Nat Protoc 2013; 8: 1551-1566.



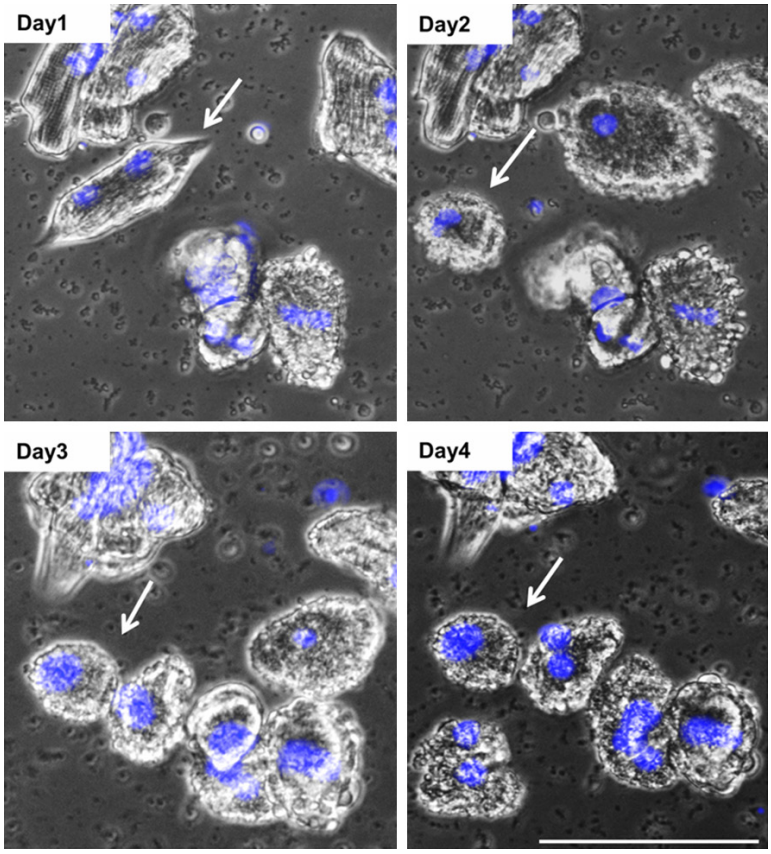
Supplementary Figure 1. Transfection efficiency of adult cardiomyocytes. Mock transfected (cel-miR-67) cardiomyocytes at day 1, Cardiomyocytes transfected with fluorescent labelled microRNA (Dy547-cel-miR-67) at day 1, 2, and 3 post transfection; Scale bar = 400 um. Cardiomyocytes transfected with control (cel-miR-67) and cytotoxic siUbC; Scale bar = 500 um.



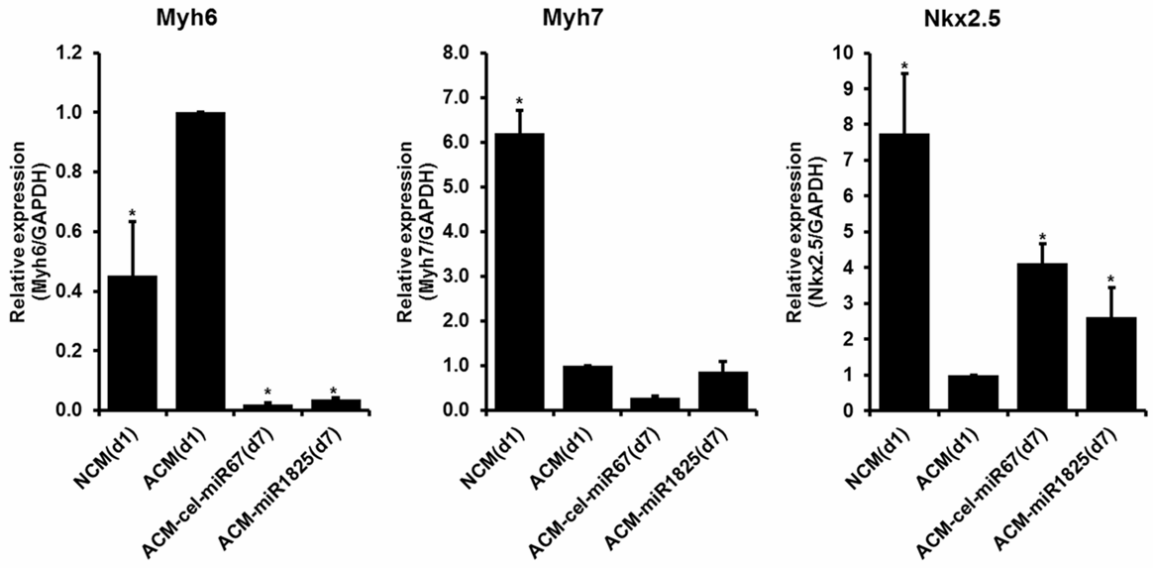
Supplementary Video 1. Video of adult cardiomyocytes 7 days post plating and transfection. Live cell imaging video; continuous video was recorded for 25 seconds showing beating cardiomyocytes.



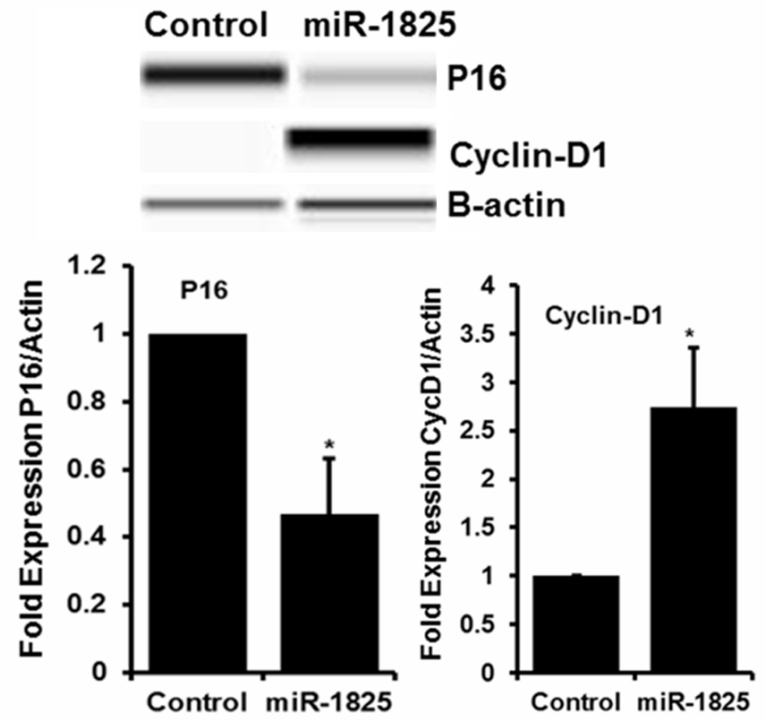
Supplementary Video 2. Video of proliferating adult cardiomyocytes transfected with miR-1825. Live cell imaging video; an image was taken at an interval of 15 minutes for 20 hours between day 3 and day 4. Color image shows a cardiac cell (green TnI) and its nucleus (Blue DAPI) at the end of the experiment.



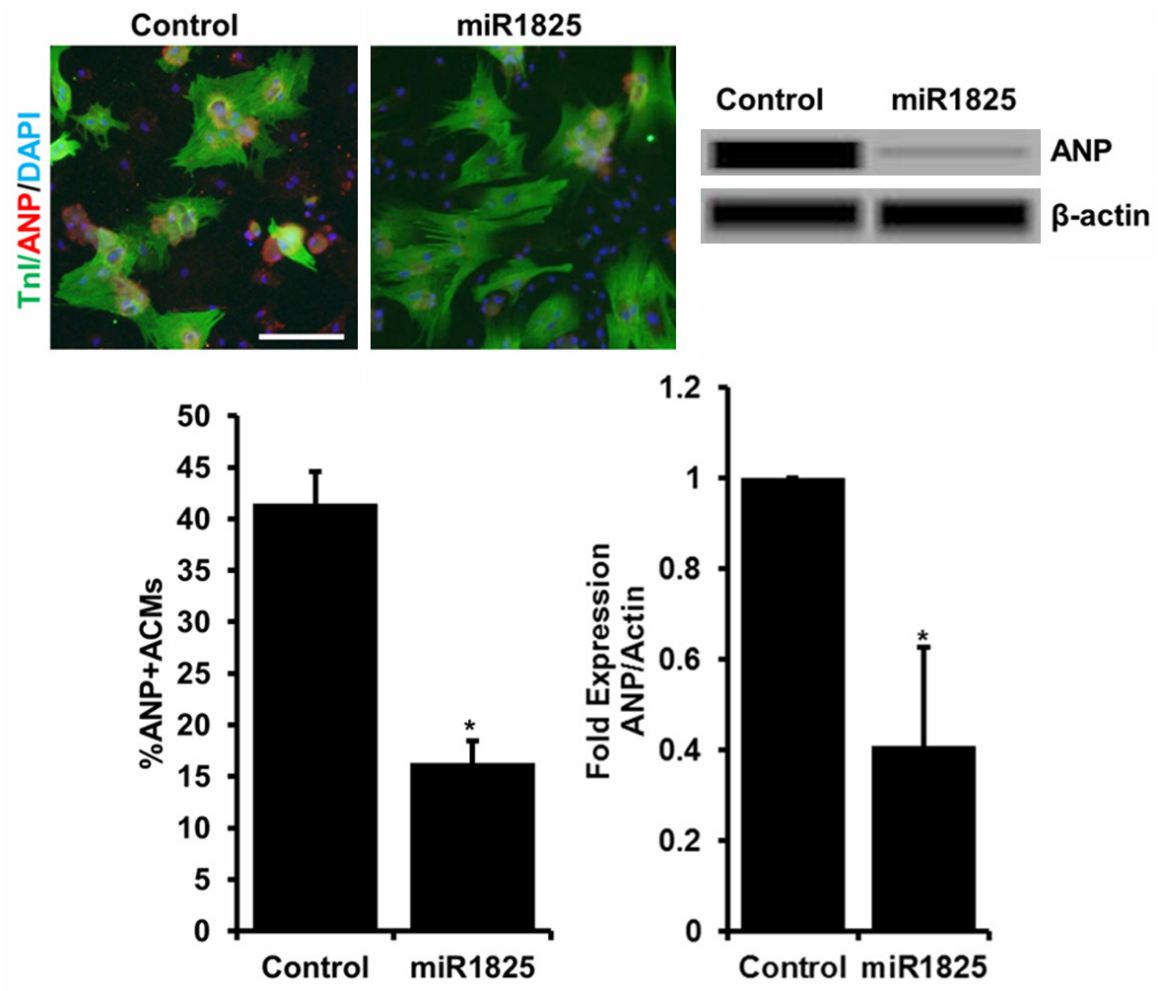
Supplementary Figure 2. Live cell imaging showed dividing cells. Individual cells were imaged everyday post transfection to capture dividing cardiomyocytes. White arrow shows a cardiomyocyte dividing into two between day 2 and day 3 post-transfection. DAPI (blue) denotes nucleus. Scale bar = 100 μm.



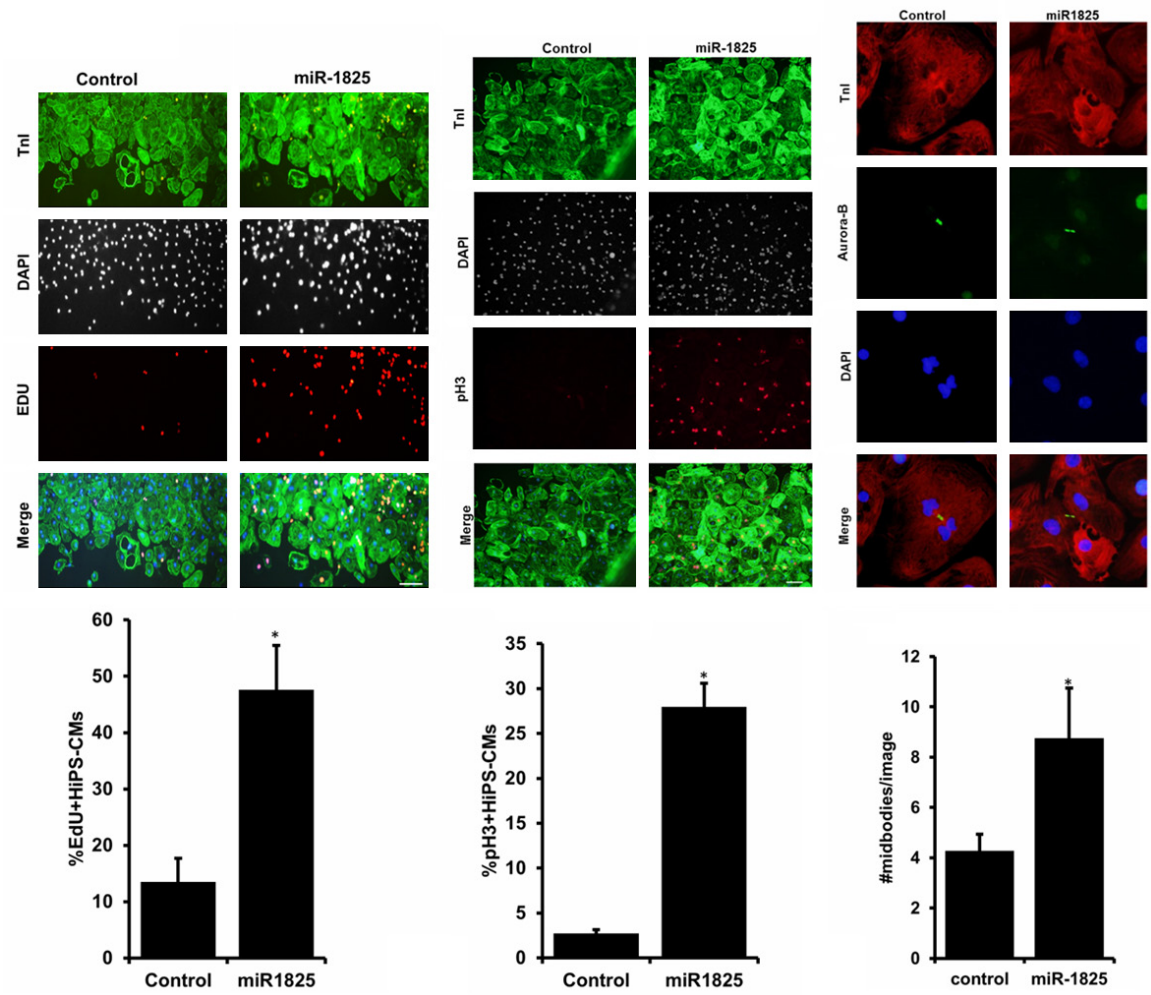
Supplementary Figure 3. Genes associated with cardiac differentiation are regulated in cultured adult cardiomyocytes. Myh6 (alpha MHC), Myh7 (beta MHC), and Nkx2.5 expression levels were measured using qRT-PCR. Fold change calculated after normalizing with GAPDH expression. N≥3, *P<0.05.



Supplementary Figure 4. Cell cycle genes are regulated by miR1825. Expression of P16 and CyclinD1 were measured by Wes Protein simple. Fold change calculated after normalizing with β-actin expression. N≥3, *P<0.05.



Supplementary Figure 5. miR-1825 prevents adult cardiomyocyte hypertrophy. Hypertrophy in ACMs was measured using ANP antibody (red). TnI (green; cardiac marker), DAPI (blue, nucleus). N = 3, *P<0.05. Scale bar = 5 mm.



Supplementary Figure 6. miR-1825 induces proliferation of HiPS-CM. HiPS-CM proliferation was measured by EDU (DNA synthesis), pH3 (mitosis), and Aurora-B (mid-bodies). TnI (green; cardiac marker), DAPI (blue or white-pseudo color, nucleus). N = 3, *P<0.05. Scale bar = 200 μm.

miRNA	Fold
Mir190_1	-3.34
Mir183	-2.72
Mir26a	-1.4
Mir181b2	1.39
Mir181b1	1.56
rno-mir-199a	8.47
Mir199a	8.82

Genes	Fold
Ace3	3.59
Acta1	4.58
Actb_1	1.4
Actg2	1.56
Actn4	1.36
Aif1l	1.52
Ankh	1.26
AU018091	5.29
Axl	1.39
Casq1	3.77
Ccnb1	3.77
Ccnd1	1.76
Cd44	1.43
Cd82	1.56
Cdca3	2.95
Cdca8	3.03
Cdkn1a	1.5
Cercam	1.9
Ckap2	3.42
Cnn1	4.6
Col16a1	2.8
Col1a1	1.48
COL4A2	1.3
Col5a1	1.61
Csrp1	1.56
EFEMP2	1.47
Ehd2	1.52
Emp3	1.51
ENSRNOG00000014343	5.52
ENSRNOG00000029843	3.84
ENSRNOG00000031029	1.73
Fads3	1.42
Fam129b	1.29
Fam50a	3.33
Fen1	2.29
Fhl1	1.27
Fkbp10	1.49
Fkbp9	1.74
FTL1	1.59
G6pd	1.34
Gpx6	1.79
Gtse1	2.61
Hmga1	2.06
Hspb1	1.26

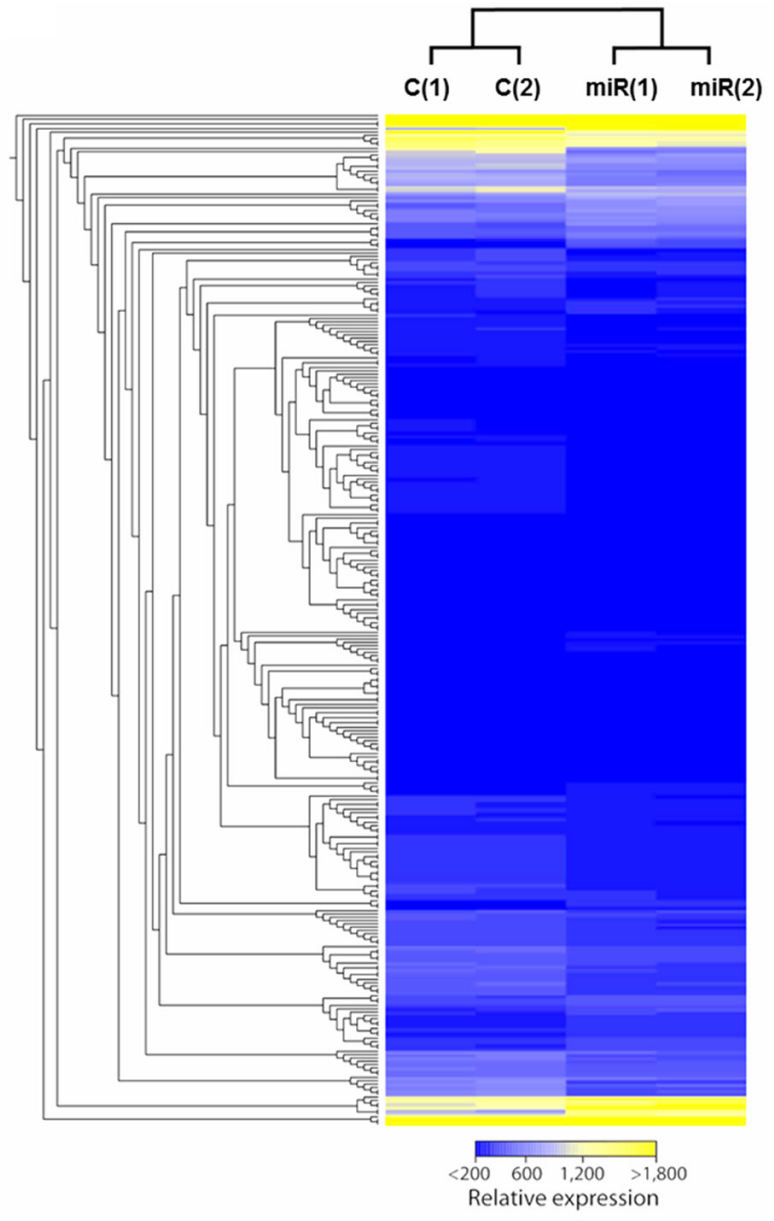
Htra1	1.9
Igsf1	3.23
Inha	3.07
Iqgap3	3.15
Itpr3	2.12
Kif20a	2.33
Kif22	2.44
Lrrc59	1.34
Ltbp2	1.27
Man1b1	1.34
Mcm3	1.8
Mcm5	2.6
Mmd	1.69
Mmp19	2.1
MT-ND2	1.82
Myl6	1.41
ND1	1.32
Nptxr	2.71
Olfml3	2.2
Pabpc1	1.18
Pcolce	1.15
PCOLCE2	2.42
Pdlim7	1.56
Phlda3	1.43
Pigu	2.05
Plk1	2.39
Pomp_2	1.67
Prc1	3.81
Ptgfrn	1.57
Ptgs1	2.19
Ptk7	1.44
Ptpla	1.75
Ptprn	2.53
PVR	1.57
RGD1561055	2.07
Rpl13_2	1.41
Rpn1	1.48
Rpn2	1.19
RRM2	2.79
Rrm2	3.1
Rtn3	1.39
S100a11	1.29
Sdc1	1.93
Sema7a	2.09
Sfrp4	4.33
Sh3bgrl3	1.59
Slc1a5	1.81
Snd1	1.3
Steap3	1.79
Stmn1_1	2.02
Sypl2	3.73
Tagln2	1.42
Tgfbi	1.37

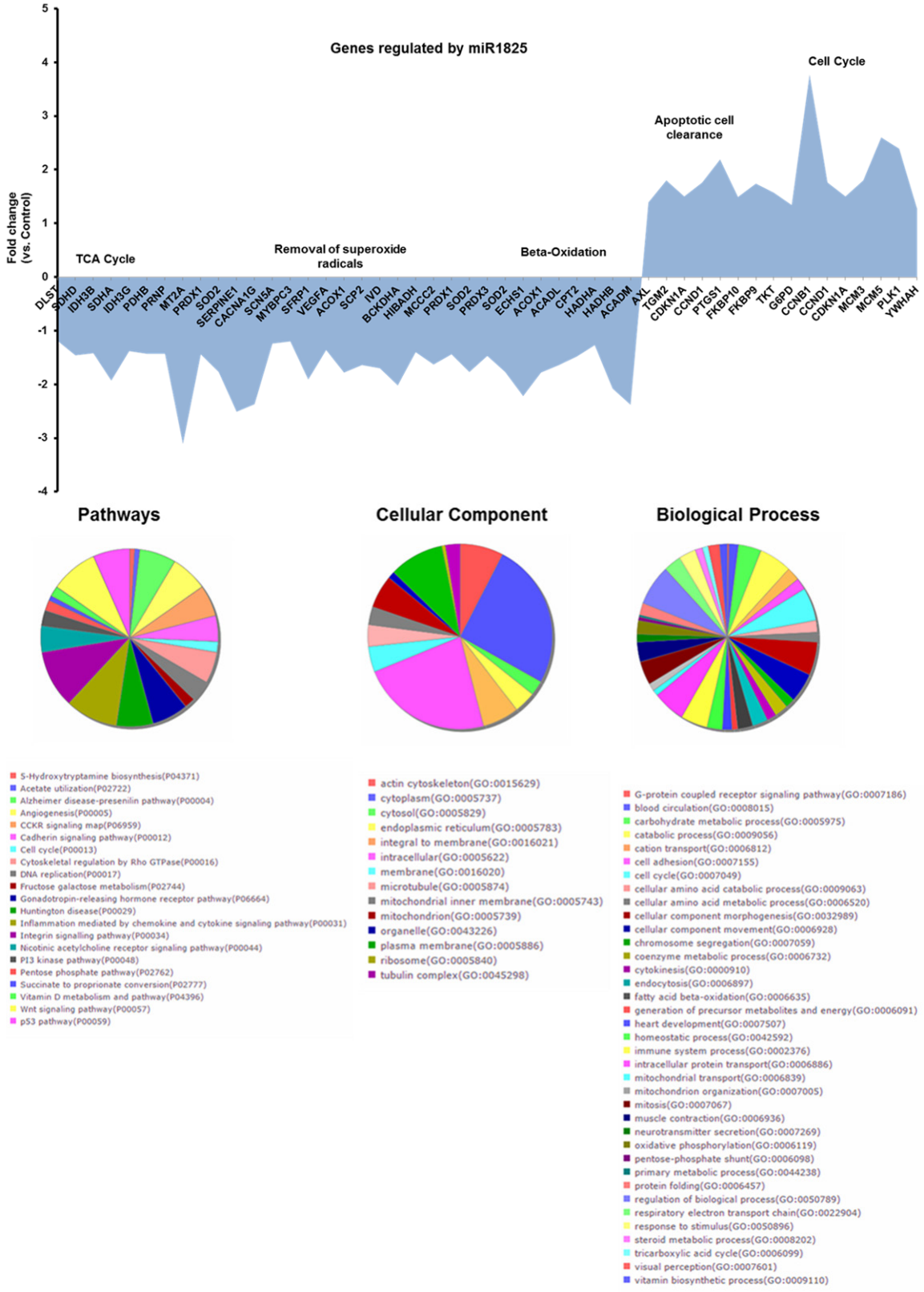
Tgm2	1.8
Tkt	1.57
Ttyh3	1.73
Tuba1c	2.98
Tubb5	1.33
Tubb6	1.39
Ywhah	1.27
A430105I19Rik	-1.5
Acad9	-1.68
Acadl	-1.64
Acadm	-2.38
Acox1	-1.78
Acsf2	-1.71
Acss2	-1.43
Adam19	-1.85
Adcy5	-1.44
Afg3l2	-1.56
Ak3	-1.76
Akap1	-1.4
Aldoc	-3.43
Ampd3	-1.47
Anapc5	-1.29
Ankrd40	-1.4
Arpc5l	-1.5
Arvcf	-3.11
Atg13	-1.38
Atp1b1	-1.29
Atp5c1	-1.35
Atp5f1	-1.68
Atp6ap2	-3.4
Atp6v1e1	-1.42
B2m	-1.44
Bckdha	-2.02
Bnip3	-1.6
CACNA1G	-2.37
Calcoco1	-1.62
Cdh2	-2.53
Celf1	-1.43
Chchd3	-1.64
Clic4	-1.12
Cluh	-1.49
Cnp	-1.83
Coq9	-1.48
Cox15	-1.56
COX6A2	-1.75
Cox7b	-1.63
Cpe	-1.45
Cpt2	-1.48
Ctsc	-1.81
CYP51A1	-1.6
Ddc	-2.83
Dhrs4	-1.82
Dkk3	-1.65

Dlc1	-1.68
Dlst	-1.19
Dnaja3	-1.65
Dnpep	-1.46
Dynll2	-1.39
Ece1	-1.61
Echs1	-2.22
Eci1	-1.89
Efnb3	-2.8
Ehd4	-1.54
Eif3h	-1.45
ENSRNOG00000017202	-2.34
Fbln5	-2.06
Fdft1	-1.94
Fgf1	-1.79
Fndc1	-2.04
Foxo4	-1.86
Fuca1	-1.6
Fzd1	-1.49
Gadd45a	-1.62
Gbas	-1.54
Gcsh	-1.64
Ghitm	-1.66
Gja1	-1.3
Gja5	-2.96
Glis2	-1.61
Gpc1	-2.28
Grb14	-1.33
H3f3c	-1.34
Hadha	-1.27
Hadhb	-2.08
Hamp	-3.71
Herpud1	-1.43
Hes1	-1.63
Hibadh	-1.4
Hk1	-1.23
Hspb8	-1.22
Idh3B	-1.42
Idh3g	-1.38
Imp4	-1.61
Insig1	-1.65
Iscu	-1.35
Itfg3	-1.8
Itga11	-2.26
Itga9	-2.09
Ivd	-1.7
Kank1	-1.25
Kif1a	-3.36
Lmcd1	-1.4
Lrpap1	-2.34
Lrrc2	-2.36
Mafk	-1.39
Map1a	-1.34

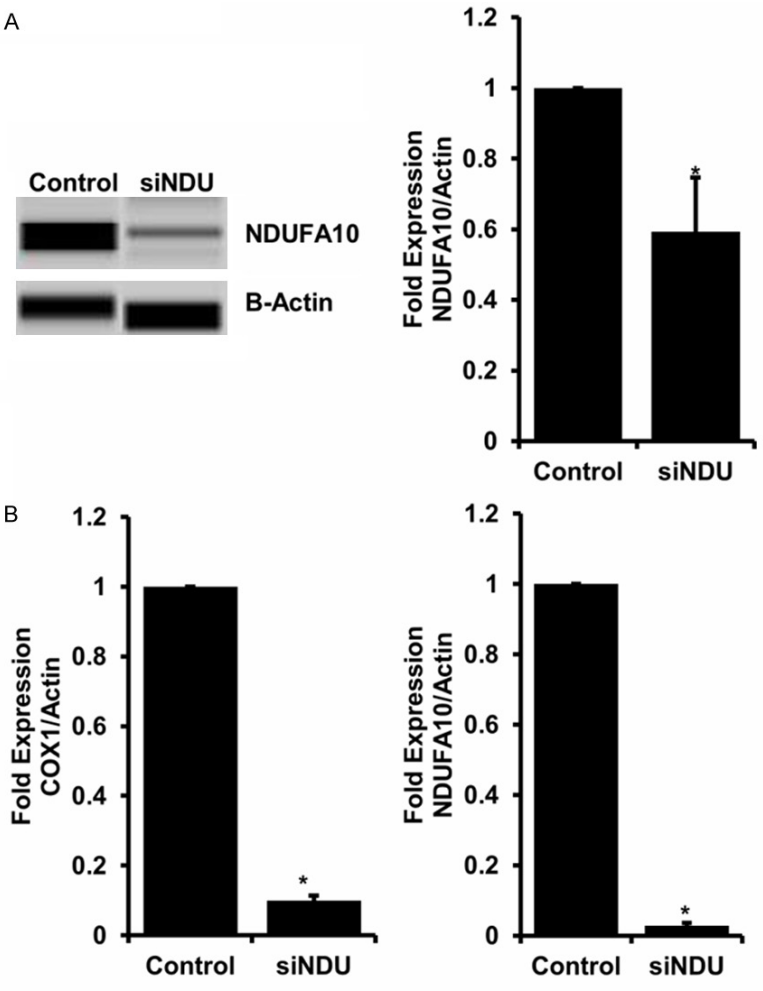
Masp1	-2.9
Mavs	-1.52
Mccc2	-1.63
Mdk	-4.43
Mfap4	-2.43
Mical2	-1.29
Mrpl45	-1.82
Msmo1	-1.45
Mt2A	-3.11
Mtch2	-1.46
Mvp	-1.46
Mybpc3	-1.2
Myh14	-1.56
Ncam1	-1.75
Ncstn	-1.66
Ndufa10	-1.34
Ndufa5	-1.51
Ndufv2	-1.58
Nek9	-1.55
NFE2L1	-1.58
Nit1	-1.58
Nmt1	-1.7
Nrbp2	-1.88
Os9	-1.78
Panx2	-2.65
Paqr8	-2.05
Pbxip1	-1.6
Pcp4	-3.11
Pdhb	-1.43
Pgrmc2	-1.41
Phc2	-1.53
Phyh	-1.69
Pigk	-1.83
Pln	-1.72
Plxnb1	-1.4
Pmpcb	-1.59
Poldip2	-1.58
Ppap2b	-1.84
Ppm1a	-1.72
Ppp1r3c	-2.03
Ppp2r1a	-1.27
Prdx1	-1.44
Prdx3	-1.47
Prelp	-3.88
Prnp	-1.43
Prss23	-1.5
Ptpra	-1.51
R3hdm2	-1.44
Rbm20	-1.39
Rbm24	-1.53
Reg3b	-6.8
RGD1309492	-1.52
RGD735029	-1.77

Rps20_2	-1.25
Sardh	-1.54
Scn5a	-1.24
Scp2	-1.64
Sdf4	-1.34
Sdha	-1.93
Sdhd	-1.46
Sema5a	-1.83
Serpine1	-2.51
Setd3	-1.49
Sfrp1	-1.91
Shroom3	-1.44
Sidt2	-1.61
Skp1	-1.34
Slc12a7	-1.51
Slc16a1	-1.69
Slc25a23	-1.88
Slc25a3	-1.25
Slc25a30	-2.12
Slc41a1	-1.6
Smpd3	-3.45
Smpdl3a	-2.11
Sod2	-1.77
Sspn	-1.91
St3gal5	-2.1
Stat3	-1.48
Stradb	-2.81
Synpo2	-1.39
Tcp11l2	-1.72
Tep1	-1.35
Tfam	-1.81
Tmem182	-1.46
Tmem63b	-2.66
Tmem66	-1.54
Tmod1	-1.64
Tnnt2	-1.22
Tns1	-1.31
Tomm40	-2.09
Trnau1ap	-1.96
Tsc22d1	-1.73
Tspyl1	-1.57
Tubg1	-2.33
Txnl1	-1.49
Ube2b	-1.48
Ube2h	-1.43
Uqcrh	-1.31
Uqcrq	-2.75
Vamp1	-2.43
Vegfa	-1.36
Vegfb	-1.6
Vnn1	-2.21
Vrk3	-2.73
Znrf2	-1.76

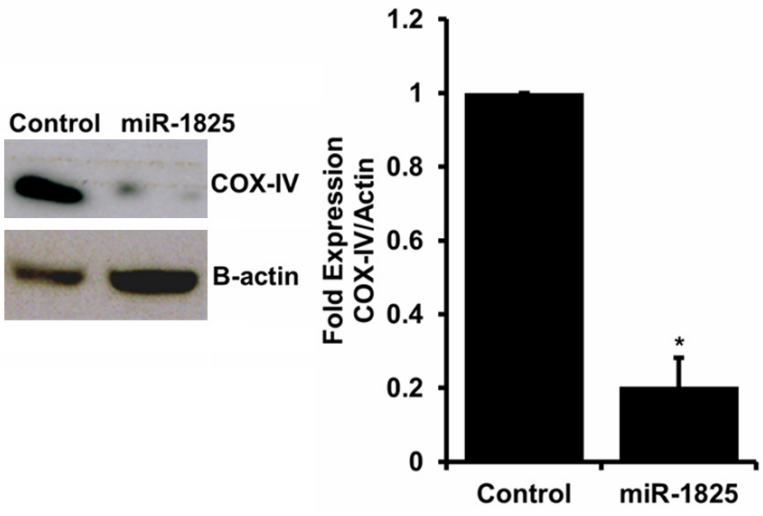

C(1)
C(2)
miR(1)
miR(2)
<200
600
1,200
>1,800
Relative expression



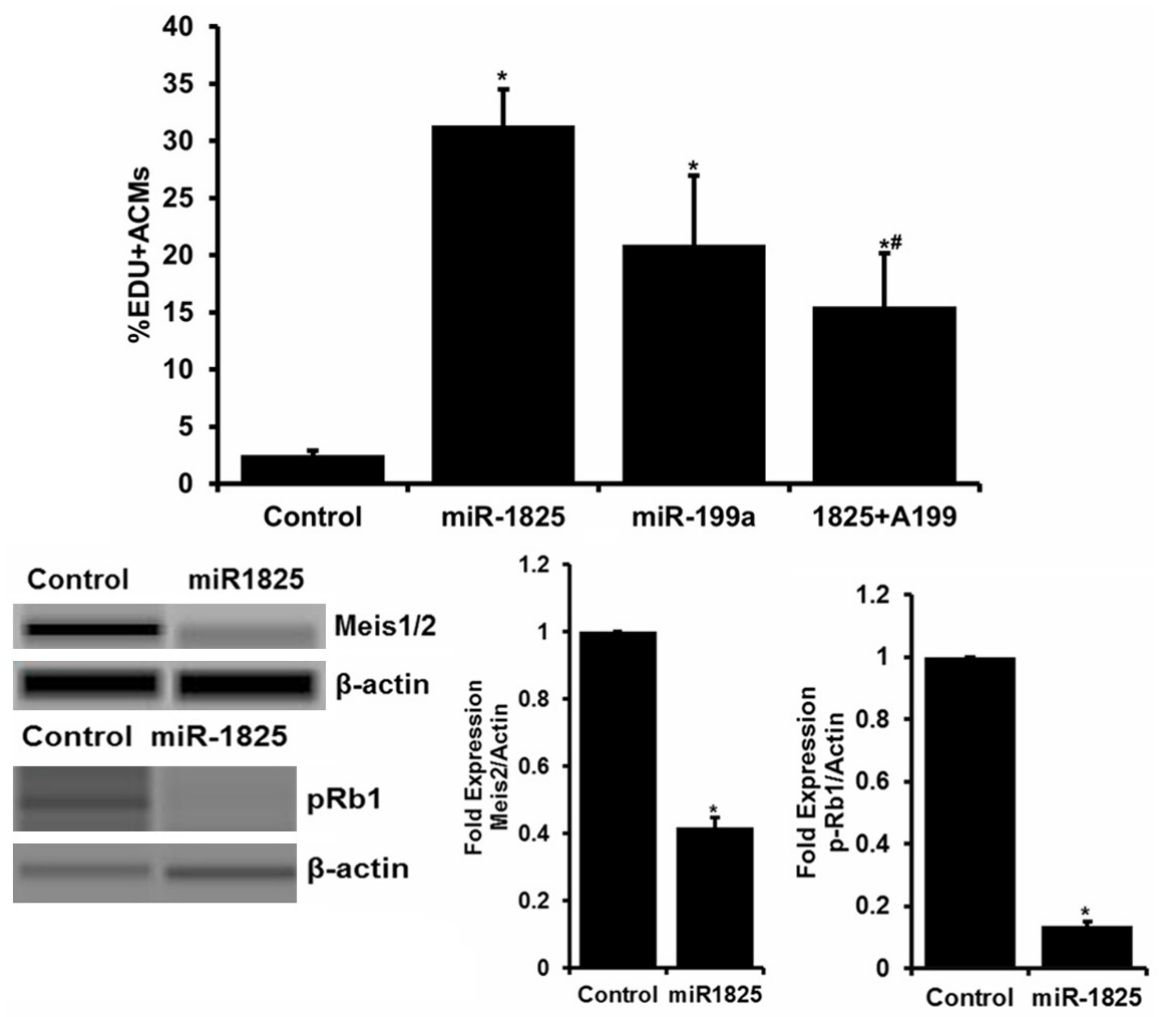
Supplementary Figure 7. Full list of genes and miRNAs regulated by miR-1825. Heatmap represents all the genes significantly regulated by miR-1825 (Relative expression represents TPM values). PANTHER analysis shows the pathways, cellular components, and biological processes regulated by miR-1825.



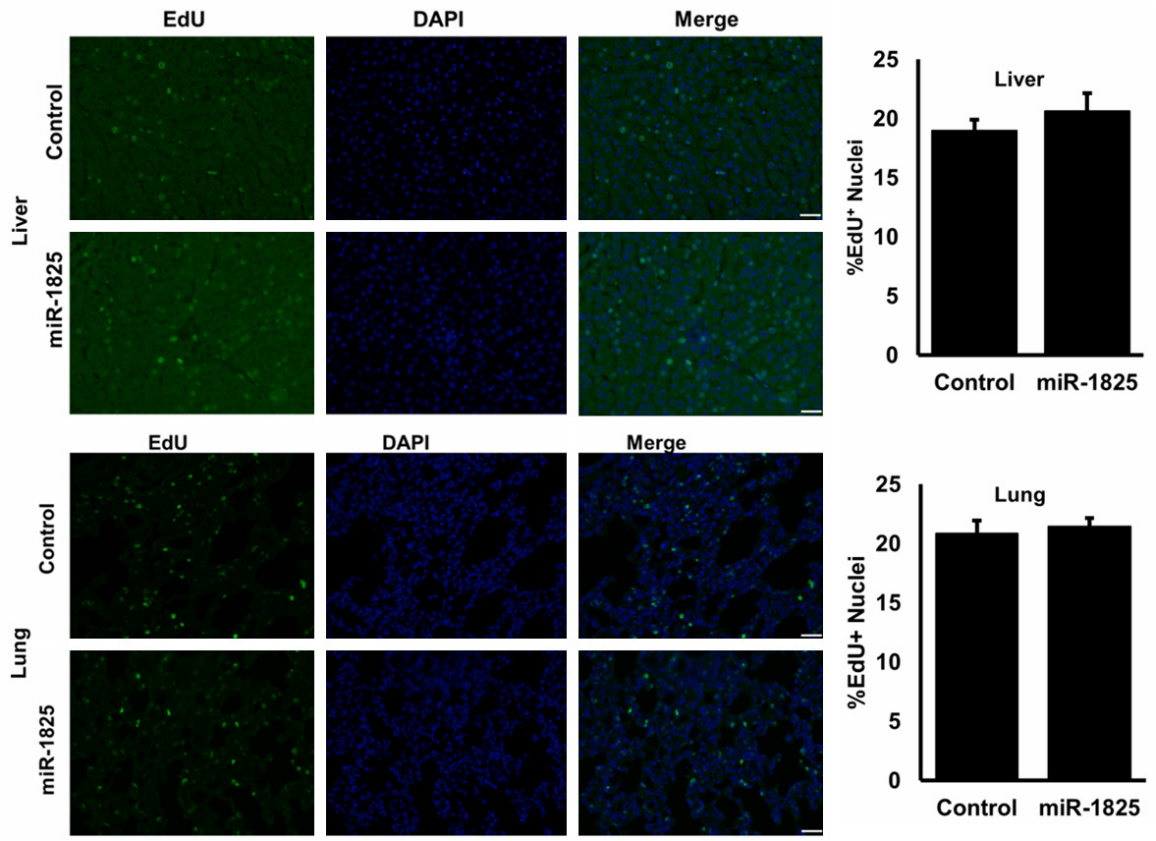
Supplementary Figure 8. siRNA mediated knockdown of NDUFA10 results in decrease in other mitochondrial genes. Efficiency of siNDUFA10 was measured at protein (A) and RNA (B) level, along with other mitochondrial gene COX1. Fold change calculated after normalizing with β-actin control. N = 3, *P<0.05.



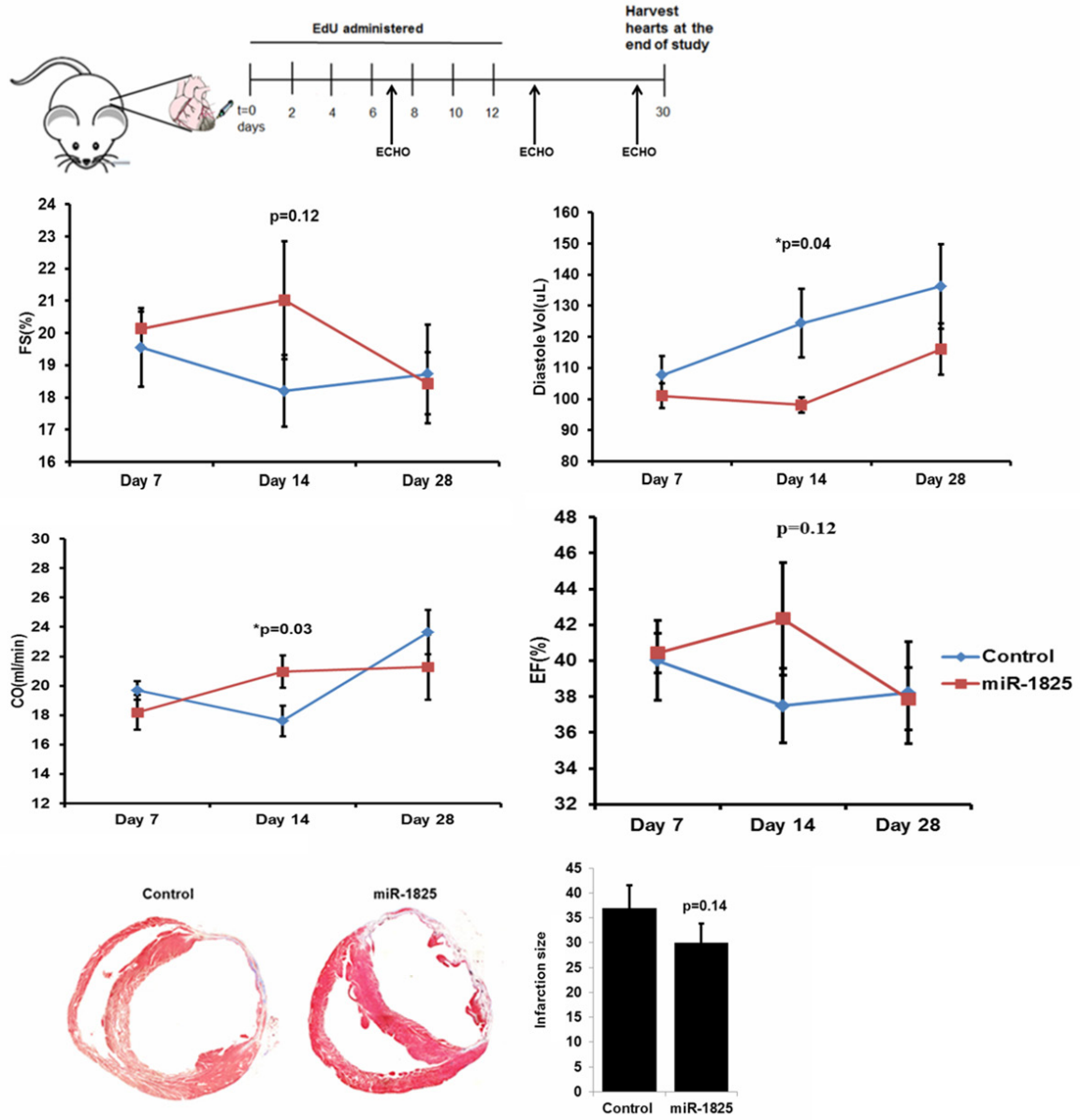
Supplementary Figure 9. miR-1825 causes reduction in COX-IV expression. COX-IV protein levels were used a marker for mitochondrial mass. Bar graph represents quantification of western blot images. Fold change calculated after normalizing with β-actin control. N = 3, *P<0.05.



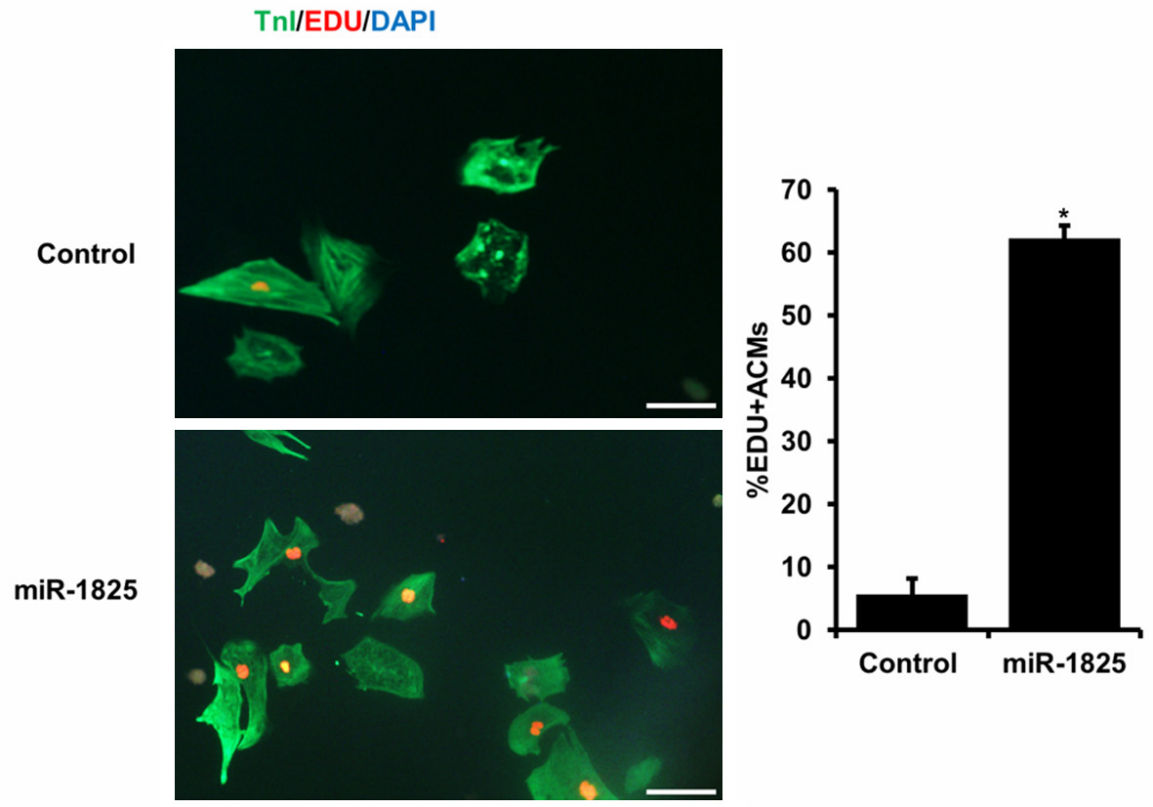
Supplementary Figure 10. miR-1825 works partly through miR-199a. EdU$^+$ cardiomyocytes were measured following transfection with Control miR-cel67, miR-1825, miR-199a, and miR-1825 together with inhibitor for miR-199a (A199) to determine relative contribution of each miRNA. N≥3; *P<0.05 (compared to control), #P<0.05 (compared to miR-1825).



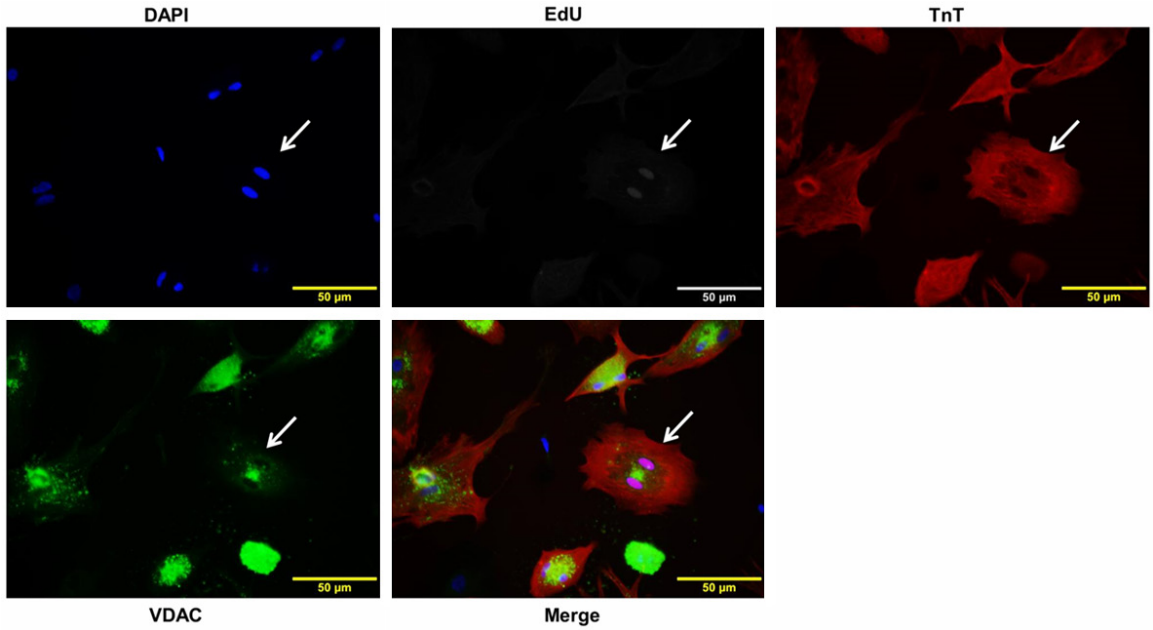
Supplementary Figure 11. miR-1825 has no effect on lung and liver cell proliferation. Proliferation measured in liver and lung tissue in neonatal (P1) rats transduced with miR-1825, intraperitoneally. EDU (green; DNA synthesis), DAPI (blue; nucleus). N = 3; NS = Not significant. Scale bar = 1 mm.



Supplementary Figure 12. Direct Injection of AAV-miR1825 does not show long-term effects. Schematic of experiment performed. Percent fractional shortening (%FS), percent ejection fraction (%EF), Cardiac Output (CO), and Diastole Volume as measured through echocardiography. Representative image for Infarction size and bar graph showing quantification of the data. N = 9 (control); N = 10 (miR-1825).



Supplementary Figure 13. miR-1825 also induces proliferation of adult cardiomyocytes from older rat heart. Adult cardiomyocytes from 17-month old rat were transfected with either control or miR-1825. EdU was used to measure DNA synthesis. *P<0.05; TnI (green) cardiac marker; DAPI (blue) for nucleus. Scale bar = 100 μm.



Supplementary Figure 14. Cardiomyocytes positive for EdU show reduced levels of VDAC compared to EdU negative cardiomyocytes. Adult Cardiomyocytes stained for VDAC (mitochondria), EdU, Troponin-T (TnT, cardiac marker), DAPI (nucleus). Scale bar = 50 μm.