Sirtuin 1 Aggravates Hypertrophic Heart Failure Caused by Pressure Overload *via* Shifting Energy Metabolism

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In vivo cardiac function evaluation by echocardiography

Animals from each group were subjected to transthoracic echocardiography at 2-, 4-, 6- weeks. Transthoracic M-mode and Doppler mode echocardiography was performed by FUJIFILM VisualSonics Vevo 3100 system. The cardiac systolic and diastolic functions were assessed using the previously described protocol [1,2]. Simpson's measurements were performed to obtain an averaged ejection fraction (EF) and fraction shortening (FS) from various parameters [1,3].

In vivo cardiac function assessment by electrocardiography

Electrical signals of the myocardium in all groups were detected by using an electrocardiogram (ECG) in Lead II (Mac Lab/4E ECG module, AD Instruments). Q amplitude, R amplitude, T amplitude, JT interval, Tpeak-Tend interval were obtained and analyzed with software LabChart 8 of mouse model default setting from the AD instrument.

Blood pressure measurement

CODA Non-Invasive Blood Pressure (Kent Scientific Corporation) was performed to measure systolic and diastolic blood pressure. Mice were placed in the plastic cone with the tail exposed and on the heating pad to keep warm. The blood pressure was determined by a tail-cuff method as described previously [4].

Immunoblotting

Protein samples were prepared by homogenizing left ventricular tissue and mixed with lysis buffer (20 mM Tris-HCl [pH 7.5], 137 mM NaCl, 0.5% NP-40, 0.5 mM 1,4-Dithiothreitol (DTT), Complete Protease Inhibitor Cocktail [Roche, Mannheim, Germany], and Phosphatase Inhibitor Cocktail [Sigma]). Immunoblotting was performed as previously described [5,6]. Protein concentrations were measured by using the Bradford assay kit (Bio-Rad, Hercules, CA). The proteins were resolved using 10% acrylamide SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The primary antibodies used in immunoblotting were rabbit polyclonal antibody against SIRT1 (Abcam, ab12193), total rodent OXPHOS cocktail antibody

(Abcam, ab110413), rabbit monoclonal antibody against phospho-PDH-E1α (Ser[293]) (Abcam, ab177461), rabbit polyclonal antibody against PPARα (Abcam, ab24509) from MitoSciences-Abcam, Eugene, OR; PDH-E1α (Santa Cruz Biotechnology, sc-377092, source: mouse, monoclonal), PGC-1α (Santa Cruz Biotechnology, sc-13067, source: rabbit, polyclonal), Mitofusin 2 (MFN2) (Santa Cruz Biotechnology, sc-515647, source: mouse, monoclonal) from Santa Cruz, CA, phospho-AKT (Ser[473]) (Cell Signaling Technology, Cat #4060s, source: rabbit, monoclonal), AKT (pan) (Cell Signaling Technology, Cat #4691s, source: rabbit, monoclonal), PDK1 (Cell Signaling Technology, Cat #3820, source: rabbit, monoclonal), phosphofructokinase-2 (PFKFB2) (Cell Signaling Technology, Cat #13045, source: rabbit, monoclonal), phospho-PFKFB2 (Ser[483]) (Cell Signaling Technology, Cat #13064, source: rabbit, monoclonal), IDH2 (Cell Signaling Technology, Cat #56439, source: rabbit, monoclonal) and GAPDH (Cell Signaling Technology, Cat #2118, source: rabbit, monoclonal) from Cell Signaling Technology Inc. (Danvers, MA).The above antibodies were used according to protocols provided by the manufacturer.

Proteomics and Immunoprecipitation analysis

All groups were subjected to total protein extraction. For immunoprecipitation analysis, 500 μg lysate were mixed with SIRT1 antibodies at 4°C 1 hour followed by 40 μL of Protein A/G PLUS-Agarose (Santa Cruz) at 4°C overnight. Both were subjected to mass spectrometric analysis with Thermo Q-Exactive-HF-X mass spectrometer coupled to a Thermo Easy nLC 1200. Samples were analyzed against the Uniprot Mouse database. The resulting samples from each group among biological replicates (n=3) were further processed using Max Quant data analysis software. Welch's t-test was performed with the ClueGO app in Cytoscape [3]. Next, ingenuity pathway analysis (IPA, QIAGEN) was utilized to determine the involved pathway of SIRT1 associated proteins and upstream transcriptional regulators that can explain the observed alterations in response to TAC-induced hypertrophy.

Cardiomyocyte's isolation

Heparin IV (Fresenius Kabi AG, Germany) for anticoagulation was given by intraperitoneal injection with 1000 units/kg 10 minutes before the experiment [5,7]. All mice groups underwent anesthesia with 2%-3% isoflurane and 100% O_2. The mice hearts were excised, cannulated by the aorta, and then connected to the cardiomyocytes' perfusion apparatus (Radnoti LLC, Covina, CA). The heart was perfused at 37 °C with perfusion buffer (pH = 7.2) containing 135 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 0.33 mM NaH_2PO_4, 10 mM glucose, 10 mM 2, 3-butane-dione monoxime, and 5 mM taurine, followed by digestion with 0.03 mg/mL Liberase (Sigma, # 5401020001) dissolved in perfusion buffer. The heart was removed and minced gently with

tweezers after completely digesting. Then, it was suspended and filtered through 100 µm filters to get the isolated cardiomyocytes.

Mitochondrial respiration and Mito fuel flex measurement

Mitochondrial respiration measurements were performed as previously described [8]. The Seahorse XF24 was used to measure the oxygen consumption rate (OCR) of isolated cardiomyocytes with equal distribution in customized Seahorse 24-well plates. We applied DMEM Medium (Seahorse Bioscience) supplemented with 1 mM pyruvate, 2mM glutamine, and 10mM D-glucose. OCR was measured using the Seahorse Bioscience XF24 Extracellular Flux Analyzer (Seahorse Bioscience) sequentially under four conditions: (1) at basal levels with no additives; (2) after adding oligomycin (1.5 µM) to inhibit ATP synthase (complex V) reversibly, indicating cellular ATP production; (3) after adding FCCP (1 µM), a mitochondrial uncoupler, to induce maximal respiration; (4) after adding Antimycin A (10 µM) to inhibit complex I, enabling calculations of non-mitochondrial respiration. The Seahorse software was used to plot the results. OCR was normalized to cell numbers per well.

A similar procedure was applied for the Mito Fuel Flex test except that OCR was measured using the Seahorse Bioscience XF24 Extracellular Flux Analyzer (Seahorse Bioscience) sequentially under two conditions: (1) after adding UK5099 (8 µM), an inhibitor of glucose oxidation pathway; (2) after adding Etomoxir (4 µM), an inhibitor of long-chain fatty acid oxidation to measure glucose dependency. The reverse order of inhibitors was used to measure fatty acid dependency.

Substrate dependency and capacity was calculated following this equation:

Dependency % = [(Baseline OCR - Target inhibitor OCR) / (Baseline OCR – All inhibitors OCR)]*100

Capicity % = {1-[(Baseline OCR - Other inhibitors OCR) / (Baseline OCR – All inhibitors OCR)]}*100

ROS measurements

Freshly frozen and non-fixed left ventricles were sectioned by cryostat in 5 µm thickness at -20°C. Sections were incubated with 1 µM MitoSOX Red (Thermo Fisher Scientific #M36008, Waltham, MA) for 10 min at 37°C to measure mitochondrial reactive oxygen species (ROS) production. The slides were then washed with 1XPBS 3 times and counterstained with 5 µg/mL wheat germ agglutinin (WGA), Alexa Fluor 488 conjugate (Thermo Fisher Scientific, #W11261, Waltham, MA) for 10 min at 37°C. After that, the slides were washed with Hank's balanced salt solution (HBSS)

twice. Images were detected by fluorescence microscopes (excitation at 510, emission at 580 nm) [5,8].

Myocardial histology

Fresh left ventricular tissues were fixed immediately in 4% buffered paraformaldehyde and then were paraffin-embedded and sectioned for staining with hematoxylin and eosin (H&E) and Masson's Trichrome. Images were taken with Keyence BZ-X710 All-in-One Fluorescence Microscope under 20X and 40X objective magnification. Cellular morphology and fibrosis were analyzed with FIJI Image J [9].

mRNA analysis by real-time PCR

The total left ventricular RNA was purified using RNAeasy mini kits (Qiagen Inc, Valencia, CA) following the manufacturer's protocol. The total mRNA was quantified with a spectrophotometer, and cDNA was synthesized using the ThermoScriptTM RT-PCR system (Invitrogen, Eugene, OR) at a concentration of 50 ng RNA/1 μL cDNA. Relative genes expression content was examined by applying real-time PCR using specific primers with the Thermofisher QuantStdudio5 real-time PCR system and iQ^{TM} SYBR Green Supermix from the Bio-Rad. The system for evaluating transcriptional level used 95°C for denaturing, 60°C for annealing, and 68°C degrees for an extension. A housekeeping gene GAPDH was run in each assay. The $\Delta\Delta C_T$ method and Thermo Fisher QuantStdudio5 real-time PCR system software was used to analyze data. Primers for *Sirt1, PPARα, PGC-1α, IDH2, Myh6, Myh7, Npaa, Npbb, Collagen 1α1, Fibronectin, CTGT, GAPDH* were listed in Supplement.

Transmission electron microscope and mitochondria dynamics analysis

Mice hearts were perfused and rapidly immersed in McDowell's Trump Fixative (Electron Microscopy Science) after excising at 4°C for 48 hours. Fixed tissues were trimmed to 1mm^3 in size and were stained with osmium tetroxide (OsO_4) and embedded in 100% Epon. The Epon block was semithin sections to further stain with 1% toluidine. The samples' preparation of TEM was performed as previously described [10]. At least 5 randomly selected electron micrographs of longitudinally arranged cardiomyocytes from each sample were examined. The images were analyzed using FIJI Image J to quantify the following parameters: mitochondrial surface area (μm^2), perimeter (μm), Feret's diameter (the longest distance (μm) between any two points within selected mitochondria), and aspect ratio (the ratio of width to height).

Isocitrate dehydrogenase activity assay

Isocitrate dehydrogenase (IDH) activity was determined for NADP+ dependent isoforms, IDH1 and IDH2, using a colorimetric IDH Assay kit from Abcam (ab102538). The left ventricles of Sirt1$^{f/f}$ and icSirt1$^{-/-}$ with sham and 6 weeks of TAC were removed and homogenized in assay buffer normalized by tissue weight. In duplicate, heart lysates (75 µg) and NADPH were loaded into 96 well clear plates and incubated with NADP+ and Isocitrate substrate. Endpoint results were used to calculate the amount of NADPH (20 mins) in each well from a standard curve. IDH activity was determined using the following equation:

IDH activity = (Amount of NADP/ (Time of reaction x Volume in well)) x dilution factor

References

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Supplemental Tables

Table S1: Echocardiographic measurements of mouse hearts functions under sham operations or 6 weeks of transverse aortic constriction (TAC) surgery in Sirt1$^{f/f}$ and icSirt1$^{-/-}$ C57BL/6J mice.

Groups / Parameters	Sham		2 weeks of TAC		4 weeks of TAC		6 weeks of TAC	
	Sirt1$^{f/f}$	icSirt1$^{-/-}$	Sirt1$^{f/f}$	icSirt1$^{-/-}$	Sirt1$^{f/f}$	icSirt1$^{-/-}$	Sirt1$^{f/f}$	icSirt1$^{-/-}$
IVS;d (mm)	1.10 ± 0.2	1.04 ± 0.11	1.19 ± 0.16	1.19 ± 0.11	1.11 ± 0.13	1.07 ± 0.10	1.05 ± 0.13	1.15 ± 0.24
IVS;s (mm)	1.60 ± 0.29	1.51 ± 0.14	1.66 ± 0.26	1.73 ± 0.23	1.48 ± 0.28	1.53 ± 0.14	1.29 ± 0.15*#	1.63 ± 0.29
LVID;d (mm)	3.27 ± 0.37	3.58 ± 0.13	3.10 ± 0.75	3.33 ± 0.31	3.44 ± 0.06	3.26 ± 0.40	3.18 ± 0.58	3.35 ± 0.41
LVID;s (mm)	1.89 ± 0.23	2.12 ± 0.15	1.98 ± 0.70	2.06 ± 0.28	2.54 ± 0.14*#	1.97 ± 0.22	2.43 ± 0.52*	2.00 ± 0.37
LVPW;d (mm)	1.25 ± 0.23	1.13 ± 0.15	1.22 ± 0.32	1.29 ± 0.3	1.14 ± 0.19	1.16 ± 0.23	1.25 ± 0.34	1.21 ± 0.25
LVPW;s (mm)	1.72 ± 0.27	1.73 ± 0.18	1.61 ± 0.34	1.89 ± 0.28	1.43 ± 0.14*	1.64 ± 0.14	1.52 ± 0.33	1.77 ± 0.28
Heart Rate (min^{-1})	413.78 ± 24.85	408.89 ± 17.26	400.38 ± 27.42	425.29 ± 24.73	419.75 ± 37.54	418.63 ± 35.97	434.5 ± 28.44	440.33 ± 36.09

IVS;s, Interventricular septum (systolic); **IVS;d**, Interventricular septum (diastolic);**IVRT**, Isovolumic relaxation time; **IVCT**, Isovolumic contraction time; **LVID;s**, Left ventricular internal diameter (systolic); **LVID;d**, Left ventricular internal diameter (diastolic); **LVPW;s**, Left ventricular posterior wall (systolic); **LVPW;d**, Left ventricular posterior wall (diastolic); N=8, *$p<0.05$ *vs.* Sirt1$^{f/f}$ sham, respectively, #$p<0.05$ vs. icSirt1$^{-/-}$ under TAC-induced stress. *P* value was determined by two-way ANOVA with Tukey's *post-hoc* test.

Table S2: Hemodynamic parameters of working heart perfusion system

Groups / Parameters	Sham		TAC	
	Sirt1$^{f/f}$	icSirt1$^{-/-}$	Sirt1$^{f/f}$	icSirt1$^{-/-}$
HR (min^{-1})	353.33 ± 17.95	375 ± 17.08	354.17 ± 10.17	368.33 ± 30.78
CF (ml min^{-1})	2.52 ± 0.23	2.65 ± 0.43	2.60 ± 0.24	2.62 ± 0.32
AF (ml min^{-1})	1.48 ± 0.23	1.52 ± 0.38	1.40 ± 0.24	1.38 ± 0.32
CO (ml min^{-1})	4.00	4.00	4.00	4.00

HR, heart rate; **CF** coronary flow; **AF** aortic flow; **CO** cardiac output. N=6. P value was determined by two-way ANOVA with Tukey's *post-hoc* test.

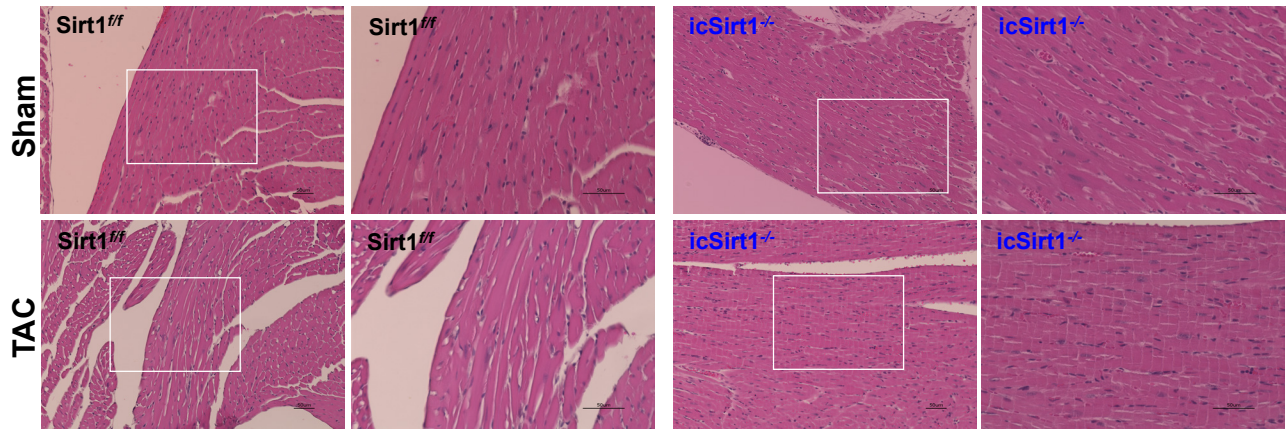## Supplemental Figures

Longitudinal section

Figure S1: Representative images of left ventricular longitudinal cross-sectional area outlined by hematoxylin & eosin (H&E).
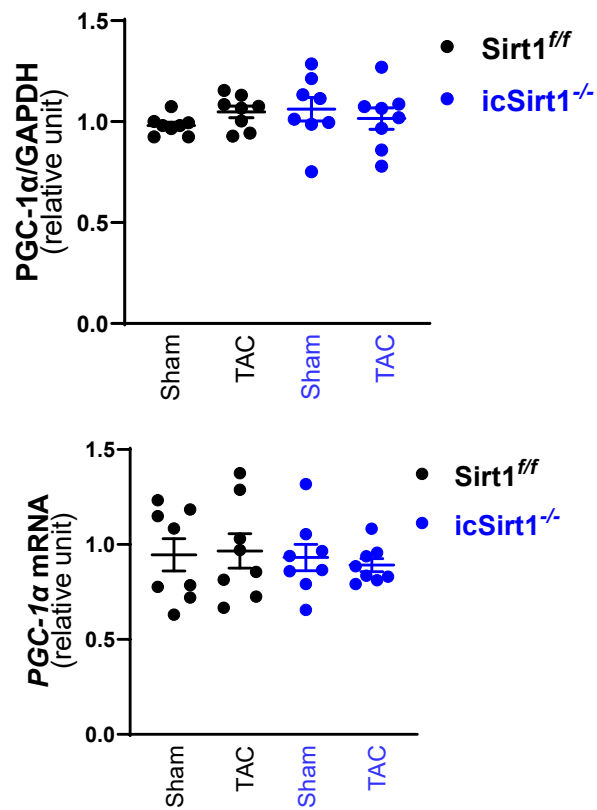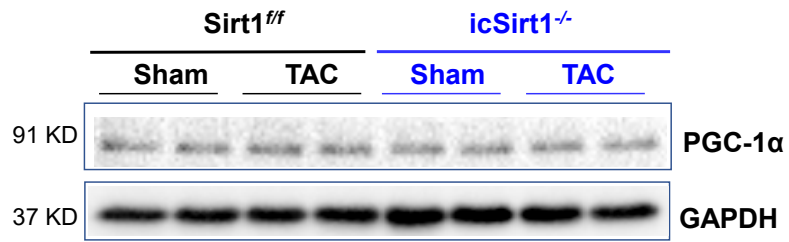
Figure S2: Protein and gene expression of PGC-1α in SIRT1$^{f/f}$ and icSIRT1$^{-/-}$ in sham and TAC conditions at the end of 6 weeks. Biological replicates N=8 for each group. *P* value was determined by two-way ANOVA with Tukey's *post-hoc* test.
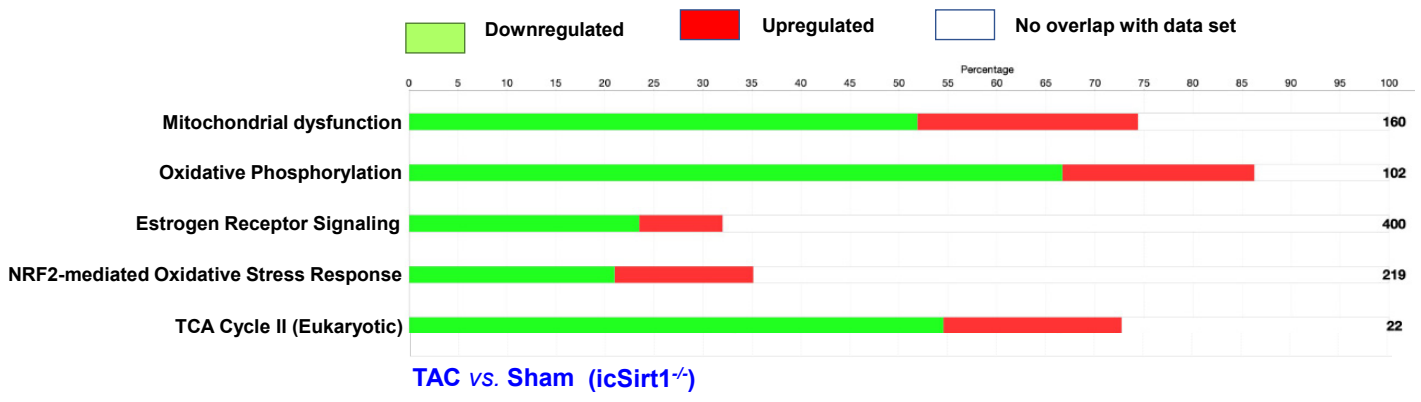
Figure S3: Ingenuity pathway analysis (IPA) enrichment analysis of proteomics of icSirt1^{-/-} 6 weeks of TAC versus sham. Green bars representing the percentage of genes in the pathway were downregulated in icSirt1^{-/-} 6 weeks of TAC versus sham. Red bars representing the percentage of genes in the pathway were upregulated in icSirt1^{-/-} 6 weeks of TAC versus sham. White bars representing the percentage of genes in the pathway were not altered in icSirt1^{-/-} 6 weeks of TAC versus sham. Biological replicates N=3 for each group.

Major Resources Table

Animals

Species	Vendor or Source	Background Strain	Sex	Persistent ID/URL
Sirt1$^{f/f}$ and icSirt1$^{-/-}$	In house	C57BL/6J	M&F	N/A

Primary Antibodies

Target antigen	Host species	Vendor or Source	Catalog#	Working concentration	Persistent ID/ URL
SIRT1	Rabbit	Abcam	ab12193	1:1000 (WB)	https://www.abcam.com/sirt1-antibody-ab12193.html
OXPHOS	Rodent	Abcam	ab110413	1:500 (WB)	https://www.abcam.com/total-oxphos-rodent-wb-antibody-cocktail-ab110413.html
p-PDHE1α (Ser^{293})	Rabbit	Abcam	ab177461	1:1000 (WB)	https://www.abcam.com/pdha1-phospho-s293-antibody-epr12200-ab177461.html
PDHE1α	Mouse	Santa Cruz Biotechnology	sc-377092	1:1000 (WB)	https://www.scbt.com/p/pdh-e1alpha-antibody-d-6
PPARα	Rabbit	Abcam	ab24509	1:1000 (WB)	https://www.abcam.com/ppar-alpha-antibody-ab24509.html
PGC-1α	Rabbit	Santa Cruz Biotechnology	sc-13067	1:1000 (WB)	https://www.scbt.com/p/pgc-1alpha-antibody-h-300
MFN2	Mouse	Santa Cruz Biotechnology	sc-515647	1:1000 (WB)	https://www.scbt.com/p/mfn2-antibody-f-5
p-AKT (Ser^{473})	Rabbit	Cell Signaling Technology	4060s	1:1000 (WB)	https://www.cellsignal.com/products/primary-antibodies/phospho-akt-ser473-d9e-xp-rabbit-mab/4060
AKT (pan)	Rabbit	Cell Signaling Technology	4691s	1:1000 (WB)	https://www.cellsignal.com/products/primary-antibodies/akt-pan-c67e7-rabbit-mab/4691
PDHK1	Rabbit	Cell Signaling Technology	3820	1:1000 (WB)	https://www.cellsignal.com/products/primary-antibodies/pdhk1-c47h1-rabbit-mab/3820
p-PFKFB2 (Ser^{483})	Rabbit	Cell Signaling Technology	13064	1:1000 (WB)	https://www.cellsignal.com/products/primary-antibodies/phospho-pfkfb2-ser483-d4r1w-rabbit-mab/13064
PFKFB2	Rabbit	Cell Signaling Technology	130454	1:1000 (WB)	https://www.cellsignal.com/products/primary-antibodies/pfkfb2-d7g5r-rabbit-mab/13045
IDH2	Rabbit	Cell Signaling Technology	56439	1:1000 (WB)	https://www.cellsignal.com/products/primary-antibodies/idh2-d8e3b-rabbit-mab/56439
GAPDH	Rabbit	Cell Signaling Technology	2118	1:1000 (WB)	https://www.cellsignal.com/products/primary-antibodies/gapdh-14c10-rabbit-mab/2118

Table S3: Quantitative real time-PCR primers

Genes	Sequences	Use
Sirt1	Forward: 5'-GCTGACGACTTCGACGACG-3' Reverse: 5'-TCGGTCAACAGGAGGTTGTCT-3'	qPCR
PPARα	Forward: 5'-CATTTCCCTGTTTGTGGCTG-3' Reverse: 5'-ATCTGGATGGTTGCTCTGC-3'	qPCR
PGC-1α	Forward 5'-ATGTGCCAGTTCTGCGGTTGTC-3' Reverse: 5'-CCATGTGCGTGTGACCTGAGAAG-3'	qPCR
Idh2	Forward: 5'- GCCCGTGTGGAAGAGTTCAA -3' Reverse: 5'- CGAAGCCCGAAATGGAC -3'	qPCR
Myh6	Forward: 5'- CCAACACCAACCTGTCCAAGT-3' Reverse: 5'- AGAGGTTATTCCTCGTCGTGCAT -3'	qPCR
Myh7	Forward: 5'CTCAAGCTGCTCAGCAATCTATTT3' Reverse: 5'GGAGCGCAAGTTTGTCATAAGT 3'	qPCR
Nppa	Forward: 5'-ACCCTGGGCTTCTTCCTCGTCTT-3' Reverse: 5'-GCGGCCCCTGCTTCCTCA-3'	qPCR
Npbb	Forward: 5'- GCCAGTCTCCAGAGCAATTCA-3' Reverse: 5'- GGGCCATTTCCTCCGACTT-3'	qPCR
Col1α1	Forward: 5'-TGTTCAGCTTTGTGGACCTC-3' Reverse: 5'-TCAAGCATACCTCGGGTTTC-3'	qPCR
Fibronectin	Forward: 5'-AGACCATACCTGCCGAATGTAG-3' Reverse: 5'-GAGAGCTTCCTGTCCTGTAGAG-3'	qPCR
CTGF	Forward 5'-GGGCCTCTTCTGCGATTTC-3' Reverse: 5'-ATCCAGGCAAGTGCATTGGTA-3'	qPCR
Gapdh	Forward: 5'-ACCACAGTCCATGCCATCAC-3' Reverse: 5'-TCCACCACCCTGTTGCTGTA-3'	qPCR