Supplemental Material

Nuclear miRNA Regulates the Mitochondrial Genome in the Heart

Das, Ferlito, Kent, Fox-Talbot, Wang, Liu, Raghavachari, Yang, Wheelan, Murphy and Steenbergen

SUPPLEMENTAL MATERIALS and METHODS

Animals: Male Sprague-Dawley rats (250-275 g, Harlan Sprague-Dawley) were used in this study. They were provided with food and water *ad libitum*. Rats were treated humanely and all experimental procedures were approved by the Institutional Animal Care and Use Committee of Johns Hopkins University.

Langendorff Rat Heart Preparation: After sufficient anesthesia was achieved with sodium pentobarbital (80 mg/kg b/w i.p. injection) (Abbott Laboratories, North Chicago, IL) and the rat was anticoagulated with heparin sodium (500 IU/kg body weight, i.v. injection) (Elkin-Sinn Inc., Cherry Hill, NJ), rat hearts were excised, cannulated, and perfused with Krebs-Henseleit buffer containing (in mmol/L) NaCl 120, KCl 5.9, MgSO4 1.2, CaCl2 1.25, NaHCO3 25, and glucose 11. The buffer was aerated with 95% O2 and 5% CO2, to give a pH of 7.4 at 37°C as described previously (1). All hearts are perfused to wash out blood and stabilize for 15 minutes, followed by perfusion with RNAlater (Qiagen, Valencia, CA), 10 ml diluted in Krebs-Henseleit buffer, for another minute.

Isolated Mitochondria Protocols: Freshly isolated mitochondria were prepared from hearts after perfused with RNAlater, by differential centrifugation (1). Briefly, at the end of perfusion, the left ventricle was dissected out and placed in Buffer A (in mM: 180 KCl, 2 EGTA, 5 MOPS, 0.2% BSA; pH: 7.25). The tissue was then digested with trypsin (0.0001 g/0.1 g tissue) in 0.7 ml of ice-cold Buffer B (in mM: 225 Mannitol, 75 sucrose, 5 MOPS, 0.5 EGTA, 2 Taurine; pH: 7.25) and finally homogenized with Buffer B with a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) using a Polytron. To further separate the heart mitochondria from other cellular components and tissue debris, a series of differential centrifugations were performed in a Microfuge 22R centrifuge (Beckman Coulter, Fullerton, CA) at 4°C. The crude pellet was then lysed with QIAzol (Qiagen, Valencia, CA).

RNA isolation: Total RNA and miRNA enriched fraction were isolated, from whole hearts, mitochondrial fraction of the hearts or cultured primary myocytes, NRVMs, using a miRNeasy kit (Qiagen, Valencia, CA), as per company's instruction. To avoid genomic DNA contamination, DNase digestion was performed using RNase free DNase kit (Qiagen, Valencia, CA), as per company's instruction.

To characterize the integrity of the isolated RNA, spectrophotometric evaluation was performed, using Nanodrop (Thermo Scientific, Wilmington, DE). All the RNA whose A_{260} (absorbance at 260 nm) value is more than 0.15 is used for further experiments. The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) was also measured in order to check the purity of the isolated RNA. For further and more accurate purity and integrity estimation of the isolated RNA, especially the RNA isolated from mitochondrial fractions, Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) was used.

miRNA Profiling: The miRNA microarray profiling was performed using Affymetrix GeneChip miRNA 1.0 arrays (Santa Clara, CA, USA) according to manufacturer's recommended protocol. Briefly, 100 ng of total RNA enriched with miRNA was labeled by polyA polymerase addition using the Genisphere FlashTag HSR kit following the manufacturer's recommendations (Genisphere, Hatfield, PA). Labeled miRNA was hybridized to the Affymetrix miRNA 1.0 array for 16 hours. Following hybridization, the array was washed and stained according to the manufacturer's protocol. The stained array was scanned using an Affymetrix GeneChip Scanner 3000. Feature extraction was performed using Affymetrix Command Console software with miRNA QC tool software. Probesets with no detectable signal in all 6 arrays were excluded from further analysis. 15 probesets were identified with 1.5 fold-change and 20% false discovery rate (FDR) from the comparison of Mitochondrial Fraction (MC) vs Total Heart homogenate (Total).

Analysis on Gene 1.0 ST arrays: 50 ng of total RNA was amplified using the Ambion Whole-Transcript (WT) Sense Target Labeling Protocol without rRNA reduction. cDNA was regenerated through a random-primed reverse transcription using a dNTP mix containing dUTP. The RNA was hydrolyzed with RNase H and the cDNA was purified. The cDNA was then fragmented by incubation with a mixture of UDG and APE1 with restriction endonucleases; and

end-labeled using the Affymetrix terminal labeling kit following the manufacturer's directions via a terminal transferase reaction incorporating a biotinylated dideoxynucleotide. 5.5 µg of the fragmented, biotinylated cDNA was added to a hybridization cocktail, loaded on an Affymetrix Rat Gene 1.0 ST GeneChip, and hybridized for 16 hours at 45 ºC and 60 rpm. Following hybridization, the array was washed and stained according to the manufacturer's protocol. The stained array was scanned using an Affymetrix GeneChip Scanner 3000, generating CEL files for each array.

The Affymetrix microarray raw data were preprocessed by Bioconductor affy package (www.bioconductor.org). Normalized gene expression values were generated from the RMA procedure which consists of three preprocessing steps: background adjustment, quantile normalization, and median polish summarization. Significance Analysis of Microarrays (2) (SAM) analysis implemented in Bioconductor "siggenes" package was applied for identifying significant changes between overexpressed miR-181c and control groups. Application of statistical filters (50% FDR) did not yield any significantly differentially expressed genes between the two groups (Online Figure IV).

qRT-PCR: After performing the purity and integrity test, the RNA was reverse transcribed using miScript Reverse Transcription Kit (Qiagen, Valencia, CA). PCR was performed using a miScript SYBR green PCR kit (Qiagen, Valencia, CA) and detected with a iQ5 detector (Bio-Rad, Hercules, CA). All reactions were performed in triplicate.

Primary culture of neonatal rat ventricular myocytes: Neonatal Rat Ventricular Myocytes (NRVMs) were isolated from the whole heart of 1-2 day old rats as described previously (3). In brief, the hearts were minced, digested with trypsin overnight at 4ºC. The day after, tissue was dissociated by stepwise collagenase treatment for a few minutes at 37ºC. Cells were pre-plated twice for 60 minutes to eliminate fibroblasts and enrich the culture for cardiac myocytes. The non-adherent myocytes were then plated at a density of 1200 cells/mm2 in plating medium consisting of 199 medium supplemented with HEPES, MEM non-essential amino acids, glucose, glutamine, 10% FBS, vitamin B12, penicillin, streptomycin, on fibronectin coated plates. The next day cells are washed and fresh medium with 2% FCS is added. The cells are maintained at 37°C in the presence of 5% CO2 in a humidified incubator.

Transfection: After isolation, myocytes were immediately transfected using an electroporator (Nucleofector, Amaxa, Gaithersburg, MD) following the protocol for neonatal rat myocytes. Transfection efficiency was monitored by GFP expression and by FACS analysis (Online Figure II).

Mitochondrial Respiration Assay: The ADP-dependence of mitochondrial respiration was assessed at 25°C in a chamber containing respiration buffer (in mM) KCl 140, EGTA 10, HEPES 20, Oxalic Acid 5, K_2HPO_4 5 and pH 7.25 and connected with a Clark-type O_2 electrode (Instech) and O_2 monitor (Model 5300, YSI, Inc) (1). After addition of transfected NRVMs ($1.5X10^6$ cells) into the air sealed chamber, the respiratory rate was measured by addition of ADP. In order to convert ADP into ATP, the cells were permiabilized with Saponin (50 μg/ml) (Sigma-Aldrich, Corp, St. Louis, MO). After addition of TMPD/Ascorbate (0.2 mM and 5 mM), the maximum respiratory rate was determined (4).

Reactive Oxygen Species (ROS) Production Assay: Hydrogen peroxide (H_2O_2) production from intact NRVMs was measured fluorimetrically by measurement of oxidation of Amplex Red to fluorescent resorufin (Life Technologies, Carlsbad, CA). After 48 hr of transfection, either with scambled RNA or miR-181c, NRVMs were washed and incubated in buffer containing 140 mM NaCl, 3.6 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 20 mM HEPES, 1.2 mM K_2HPO_4 and 11 mM glucose (pH 7.4). All incubations also contained 50 μM Amplex Red and 5 U/ml of horseradish peroxidase. The increase in fluorescence at an excitation of 544 nm and an emission of 590 nm was monitored. Standard curves were generated using known amounts of hydrogen peroxide (5).

NRVMs Preparation for Western Blot: Transfected NRVMs were lysed with RIPA buffer and protein content was measured using Bradford assay (1). Cell homogenate protein was separated by 1D gel electrophoresis. After transfer to a PVDF membrane, the membrane was incubated with antibody that recognizes proteins such as Dicer, Prohibitin, mt-COX-1, Cadherin and a-actinin from Santa Cruz Biotechnologies Inc., Santa Cruz and mt-COX2 (Life Technologies, Carlsbad, CA) in Tris-Buffered Saline (pH 7.4) with 1% TWEEN 20 (TBS-T) with 5% BSA or nonfat dry milk at 4°C overnight. Membranes were incubated with the

secondary antibody, appropriate horseradish peroxidase–conjugated IgG in TBS-T with 5% nonfat dry milk for 1 hour at room temperature. Immunoreactive protein was visualized using an enhanced chemiluminescence analysis kit (GE HealthCare**,** Piscataway, NJ).

Fluorescence In-Situ Hybridization (FISH): After isolation, NRVM were immediately platted for 2 days to attach with the plates. Mitochondria were labeled by exposing to live cell-staining solution containing mitochondrial membrane potential dye (MitoTracker Red, Invitrogen, Grand Island, NY) for 30 min (6). Cells were then fixed in acid methanol (60% methanol and 10% Acitic Acid) and allowed to dry overnight. Detection of miR-181c was performed by fluorescence in situ hybridization using locked nucleic acid probes and tyramide signal amplification (7). Cells were then hybridized with LNA microRNA probe double dig-labeled has miR-181c (Exiqon, Woburn, MA) for 1 hour at 56^{o}C followed by a series of posthybridization stringent washes with SSC buffer (Invitrogen) at 56^{o}C. After a DIG buffer wash (Roche Diagnostics, , Mannheim, Germany) , endogenous peroxidase activity of the cells was quenched with a 3% hydrogen peroxide solution followed by a TNB block (Perkin Elmer, Waltham, MA). Anti-DIG POD (Roche) was applied to the cells for 30 minutes. Finally, cells were incubated with TSA Plus Fluorescein Amplification Reagent. After a series of washes with TNT buffer, the chambers were removed and the slides were cover slipped with Vectashield with DAPI (Vector Labs, Burlingame, CA). Control slides underwent hybridization with no probe or received no TSA Plus Fluorescein Amplification reagent.

Luciferase reporter assays. 0.8X10^{5} HeLa cells were plated in triplicate wells of a 24-well plate and transfected 16 hours later with 50 ng of pGL3-control COX-1 UTR reporter construct with firefly luciferase (f-luc) and 0.5 ng of phRL-SV40 (Promega) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. 24 hours after transfection, cells were lysed and assayed for firefly and renilla (served as internal control) luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). Where indicated, control or miR-181c miScript mimics (QIAGEN) were co-transfected at 15 nM final concentration.

Online Table I. Primer Sequences for qRT-PCR

a) 12S rRNA

Forward: 5'-AAACTGCTCGCCAGAACACT-3'

Reverse: 5'-TAGGCTGAGCAAGAGGTGGT-3'

b) mt-COX1

Forward: 5'-AGCCGGGGTGTCTTCTATCT-3'

Reverse: 5'-AAAGGATTGGGTCTCCACCT-3'

c) mt-COX2

Forward: 5'-GCTTACAAGACGCCACATCA-3'

Reverse: 5'-GAATTCGTAGGGAGGGAAGG-3'

d) mt-COX3

Forward: 5'-AGCCCATGACCACTAACAGG-3'

Reverse: 5'-TGGCCTTGGTATGTTCCTTC-3'

e) ND-2

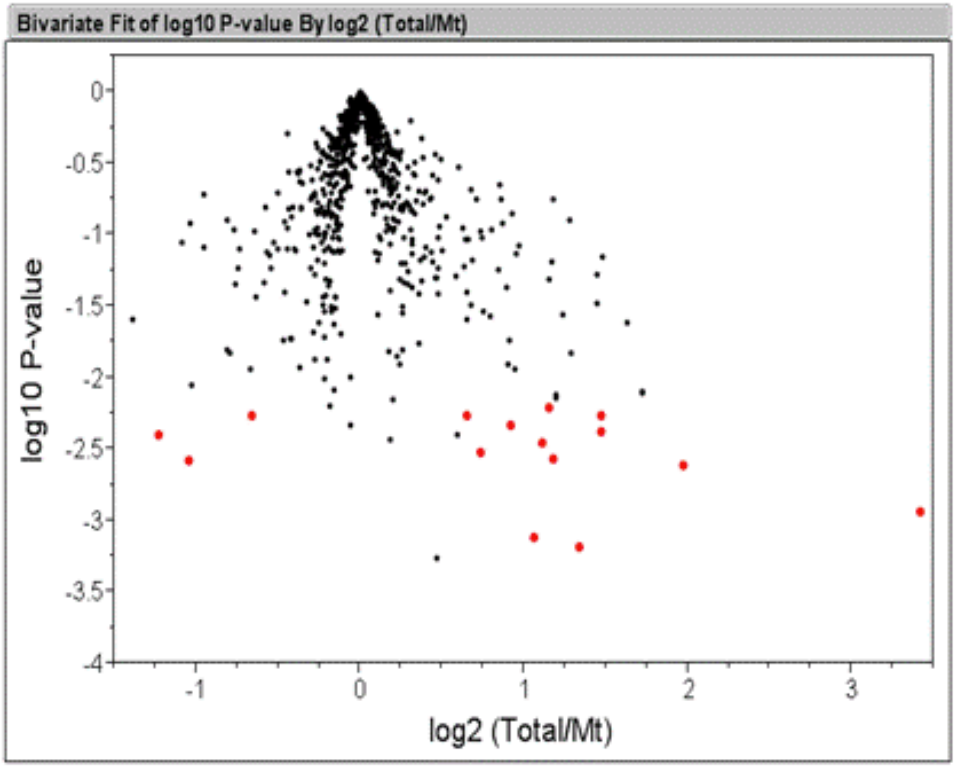
Forward: 5'-AACCCAAGCTACAGCCTCAA-3'

Reverse: 5'-GGGAATTCCTTGGGTGACTT-3'

f) ATPase8

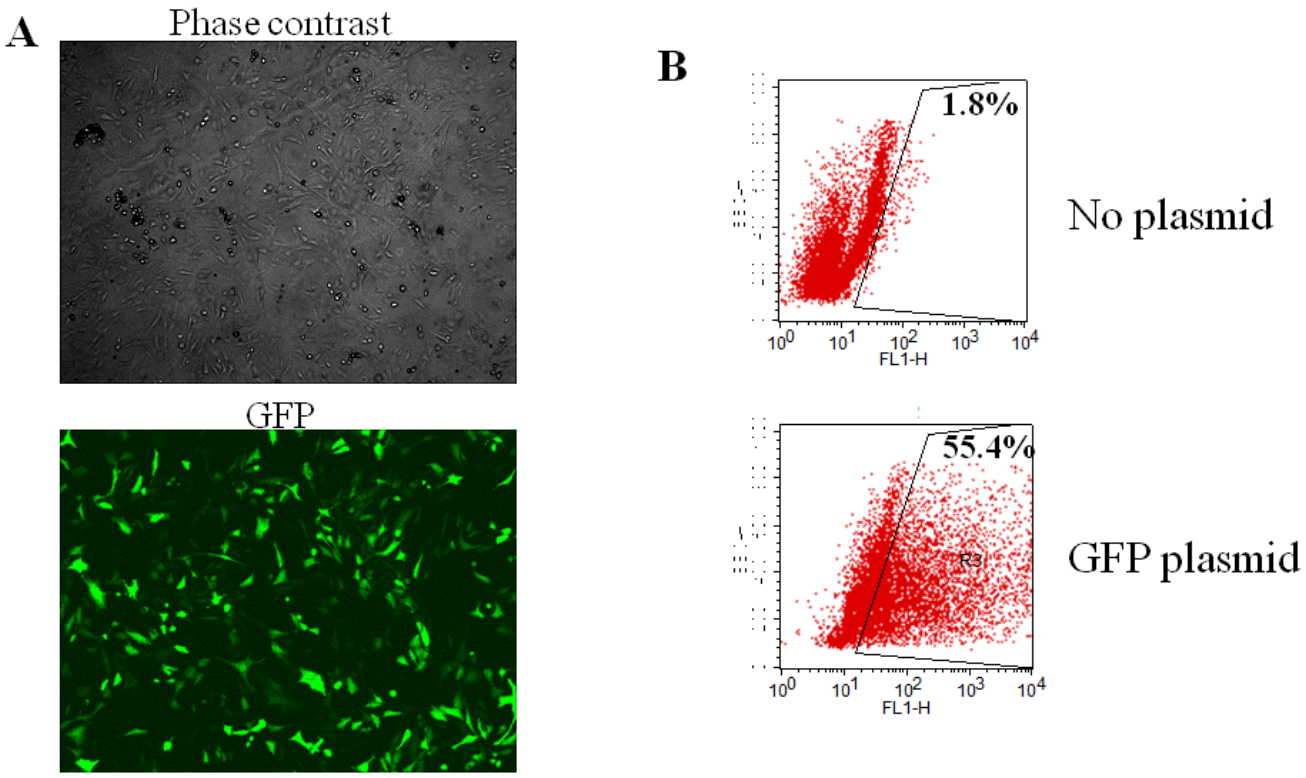
Forward: 5'-TGCCACAACTAGACACATCCA-3'

Reverse: 5'-TGTGGGGGTAATGAAAGAGG-3'



Online Figure I. Comparison between Mitochondrial (Mt) and Total heart (Total) miRNAs.

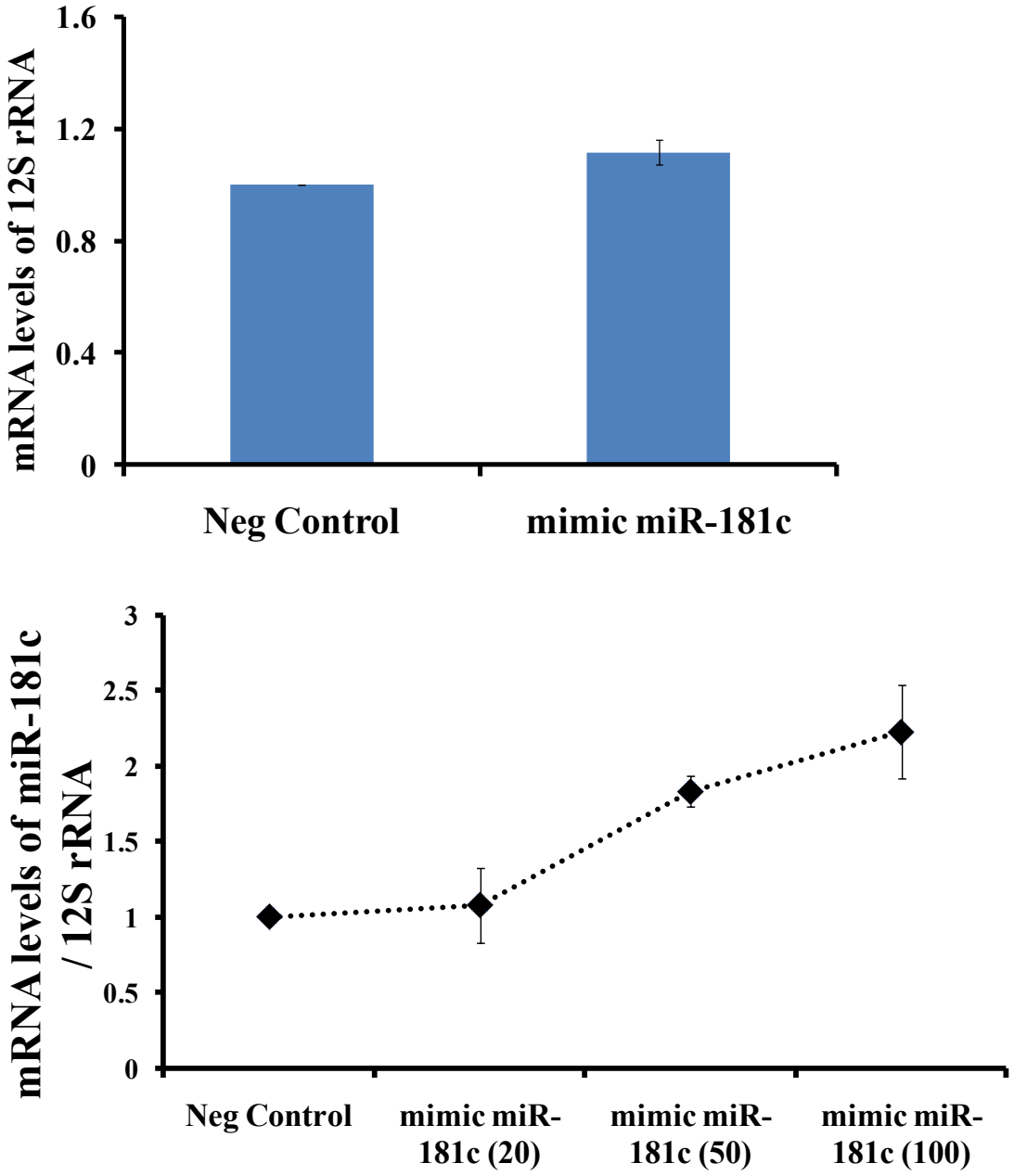
Volcano Plot representation of the two groups where 15 probes in red were selected that were considered to be expressed in the mitochondrial fraction with 1.5 fold change, 20% FDR (False Discovery Rate) and present call filtering. There are 3 miRNAs from rat, 7 from mouse and 5 miRNAs from human.



Online Figure II. Transfection Efficiency, measured by GFP expression using FACS analysis

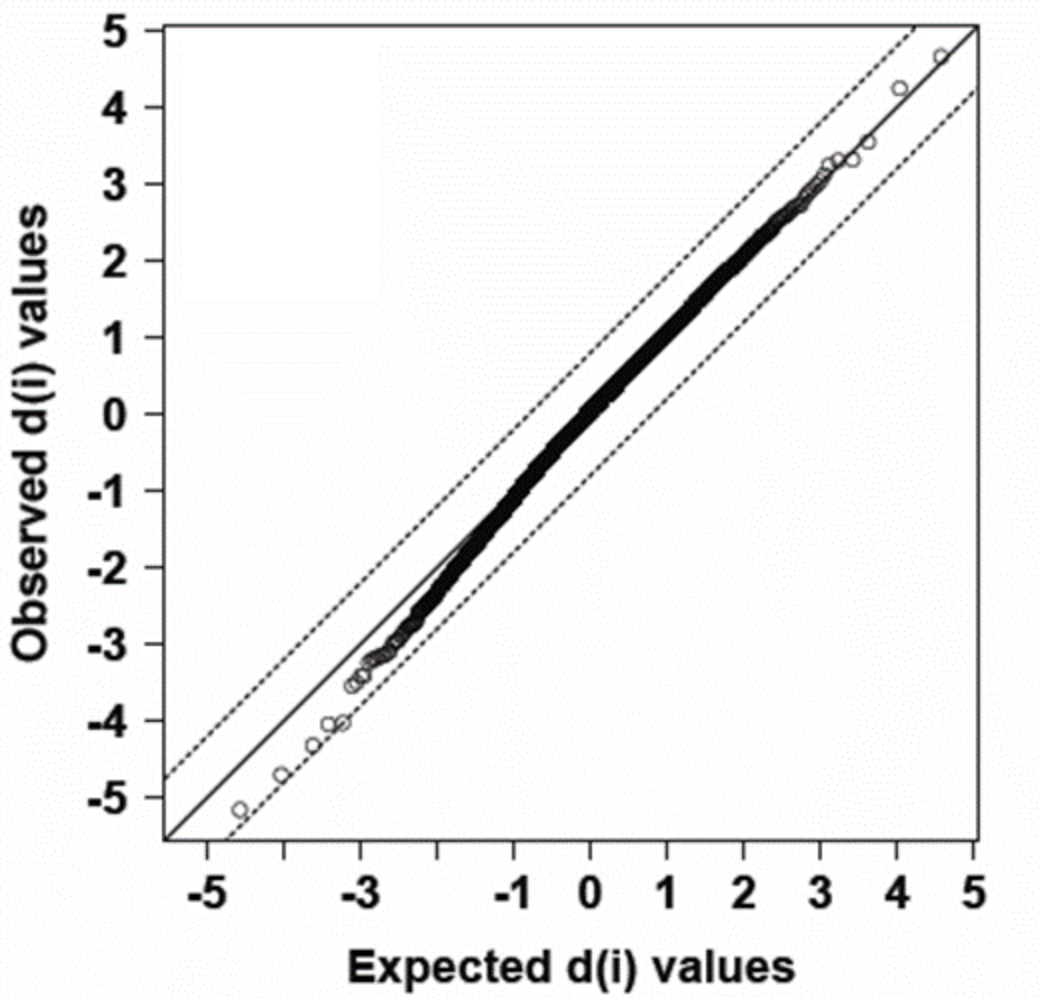
A) A field of GFP transfected myocytes and its corresponding phase contrast.

B) Representative FACS analysis of cells transfected without plasmid (upper panel) or with GFP plasmid (lower panel). Percentage of fluorescent positive cells is shown.



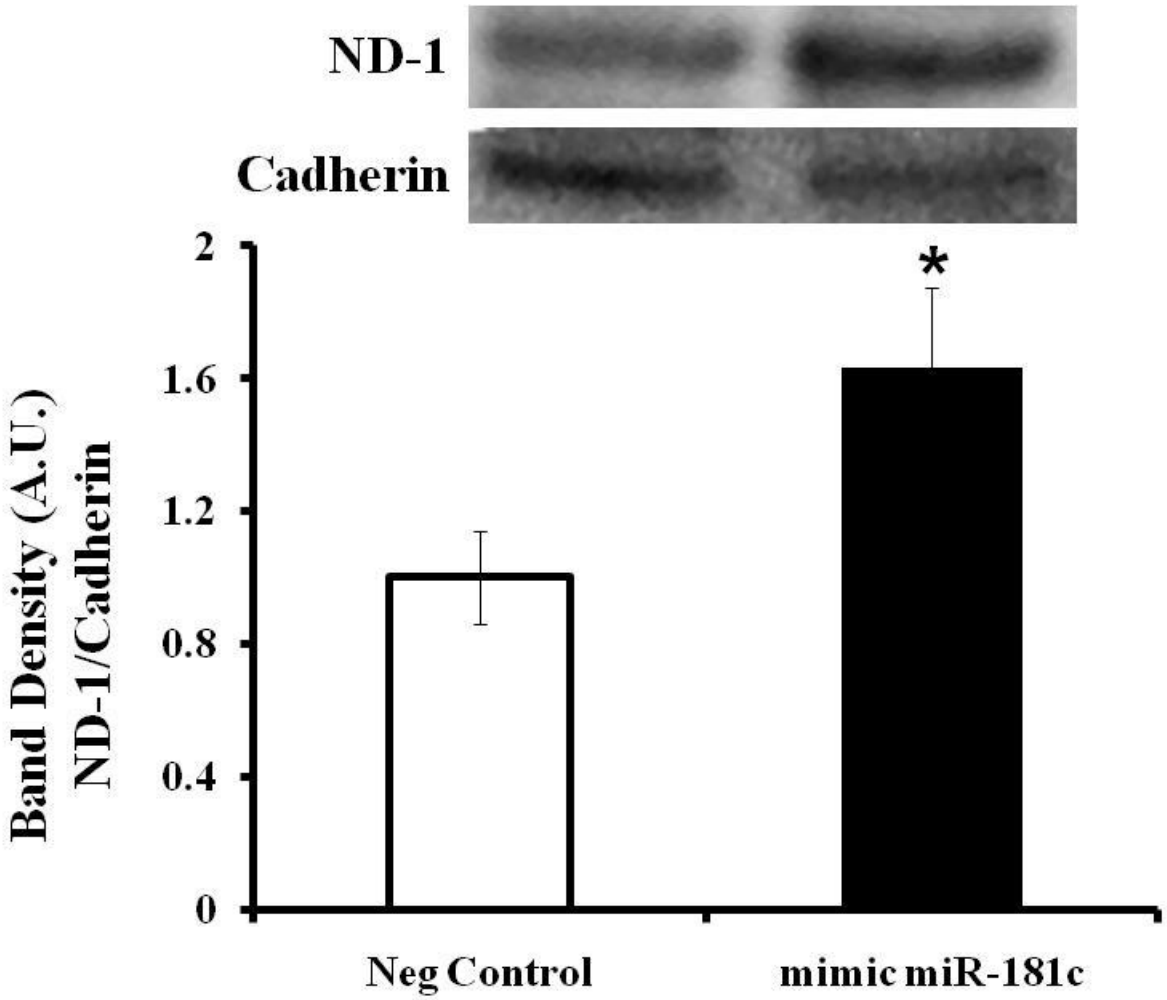
Online Figure III. Transfection of miR-181c in NRVMs

Using qRT-PCR, 12S rRNA (upper panel) mRNA expression was determined. The consistency of mRNA levels of 12S rRNA among the two groups (100 nM scrambled RNA and miR-181c transfected NRVM), makes 12S rRNA a useful internal control. In the lower panel, the dose response curve of miR-181c was analyzed. Among the three different doses (20 nM, 50 nM and 100 nM), 50 nM was picked for further experiments.



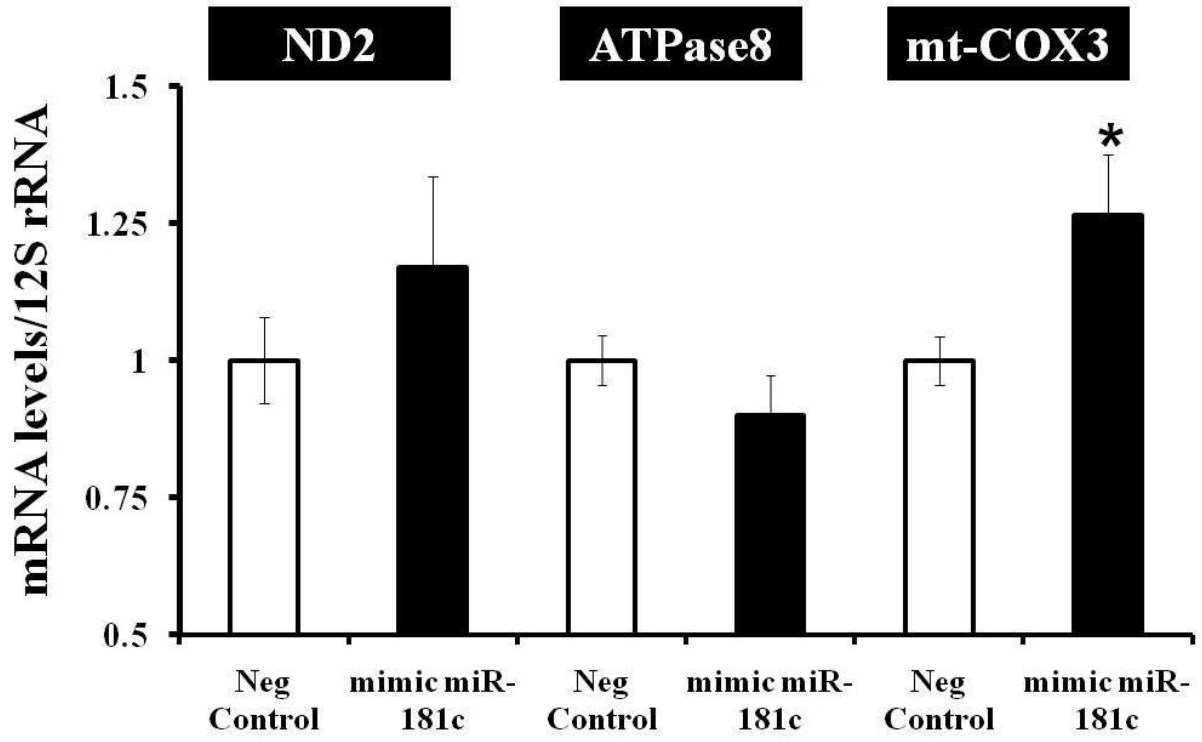
Online Figure IV. Comparison between miR-181c overexpressed and control on Rat Genome Affymetrix Gene Array

SAM Plot shows an insignificant finding on the differential expression of genes between the miR-181c overexpressed and control. The observed relative difference (di) represented in the Y-axis is almost identical with the expected relative difference (di) represented on the X-axis.



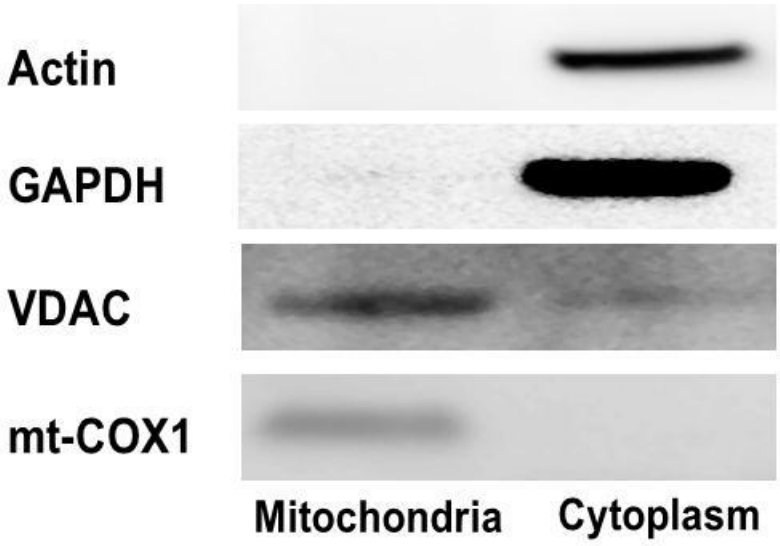
Online Figure V. Comparison of between miR-181c overexpressed and negative control transfection by Western blot Analysis.

Overexpression of miR-181c significantly increases the protein expression of ND-1 (NADH-ubiquinone oxidoreductase chain 1) compared to the negative control. Content of ND-1 was normalized to Cadherin. *****<p 0.05 vs Neg Control. Mean ± SEM (n=4) are shown.



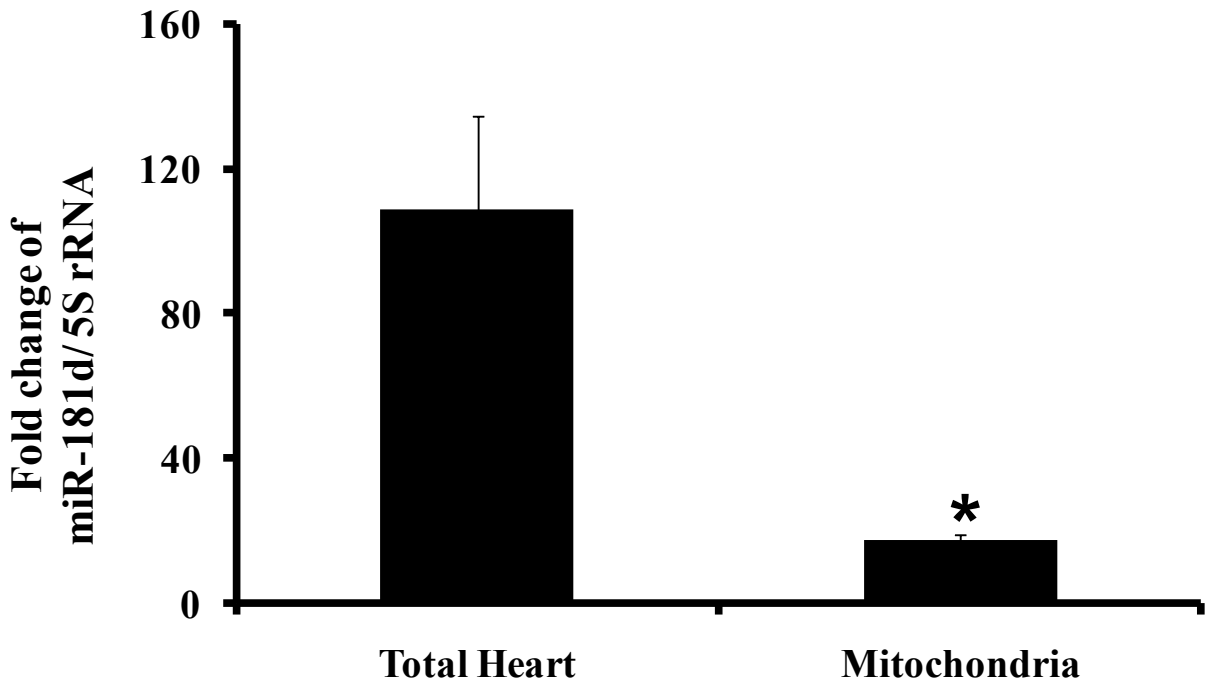
Online Figure VI. Comparison of some mitochondrial genes between miR-181c overexpressed and negative control transfection by qRT-PCR.

Overexpression of miR-181c increased ND-2 (NADH-ubiquinone oxidoreductase chain 2) and significantly increased mt-COX3 mRNA (right side pair) content. On the other hand, there is practically no change of ATPase8 mRNA content with overexpression of miR-181c (middle set) compared to negative control. Content of ND-2, ATPase8 and mt-COX3 were normalized to 12S rRNA. ***<p 0.05 vs Neg Control. Mean $\pm$ SEM (n=6) are shown.



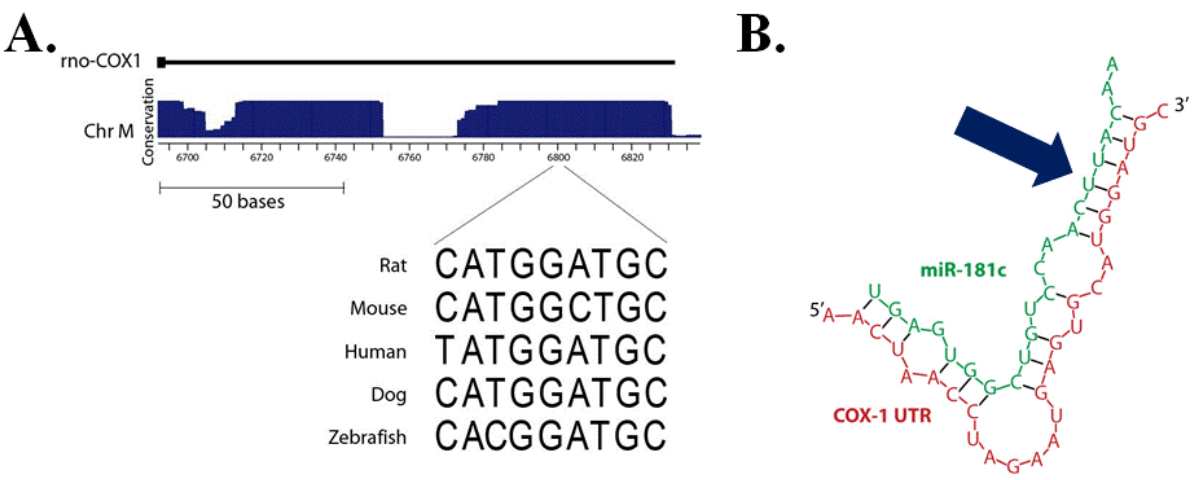
Online Figure VII. Validating the mitochondrial purity by western blot.

Western blot shows that both cytosolic proteins, Actin and GAPDH, are almost negative in our mitochondrial preparation. Additionally, our cytosolic fraction barely contains any mitochondrial protein, such as Voltage Dependent Anion Channel (VDAC) or mt-COX1.



Online Figure VIII. miR-181d is not present in the mitochondrial fraction.

qRT-PCR shows that miR-181d expression is mainly detected in the RNA derived from Total heart and not in the mitochondrial fraction. Content of miR-181d was normalized to 5S rRNA. ***<p 0.05 vs Total Heart. Mean ± SEM (n=3) are shown.



Online Figure IX. Validation of mt-COX1 as a direct target of miR-181c.

(A) From the UCSC genome browser (www. genome.ucsc.edu), we analyzed the sequence located in the 3'UTR of rat mt-COX1 (rno-COX1) in chromosome M, where miR-181c potentially binds in a complementary reverse sequence manner. The solid blue region clearly demonstrates that this sequence region in the 3'UTR of mt-COX1 is conserved across species. Below that shows the DNA sequence for 5 different species where the seed sequence of miR-181c binds. The binding site between human, rat, and dog shares 100% homology while other species have 1 nt difference compare to rat.

(B) This cartoon shows the secondary structure of the 3'UTR of mt-COX1 mRNA and miR-181c (generated from www.RNAhybrid.com). The green chain represents miR-181c and the red one represents the 3'UTR of mt-COX1. The blue arrow indicates the potential binding site of the "seed" sequence of miR-181c.

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