iScience, Volume *23*

Supplemental Information

Differential Regulation of mTOR Complexes with miR-302a Attenuates Myocardial Reperfusion Injury in Diabetes

Arun Samidurai, Ramzi Ockaili, Chad Cain, Sean K. Roh, Scott M. Filippone, Donatas Kraskauskas, Rakesh C. Kukreja, and Anindita Das

Supplemental Information

Table S1.

Parameter	Control (n=7)	I/R (n=7)	DM (n=10)	DM +I/R (n=12)	DM + I/R + RAPA (n=12)
Body Weight (Kg)	3.2 ± 0.08	3.1 ± 0.09	3.2 ± 0.059	3.41 ± 0.079	3.46 ± 0.078
Age (months)	3.4 ± 0.2	3.5 ± 0.6	5.7 ± 0.3	5.8 ± 0.4	5.8 ± 0.2
Blood glucose level (mg/dL)	162 ± 5.75	161 ± 9.78	339 ± 19.5 **	349 ± 16.8 **	337 ± 11.4 **
Heart Weight (g)	9.5 ± 0.35	9.06 ± 0.33	10.12 ± 0.31	11.25 ± 0.22	10.33 ± 0.24
Heart/Body Weight (g/Kg)	2.97 ± 0.07	2.87 ± 0.15	3.17 ± 0.11	3.39 ± 0.11	3.1 ± 0.09

Blood glucose and physiological parameters of rabbits. Related to Figure 1.
Body weight, age, blood glucose and heart/body weight ratio of control, ischemia/reperfusion (I/R), diabetes (DM), diabetes+I/R (DM+I/R) and diabetes+I/R+Rapamycin (DM+I/R+RAPA) groups at the end of the protocol. Values are mean ± SEM. All comparisons were determined using ANOVA + Bonferroni test, **$p<0.0001$ vs Control & I/R.

Table S2.

A **Oryctolagus Cuniculus miRNAs (12 Sequences) OryCun 2.0**

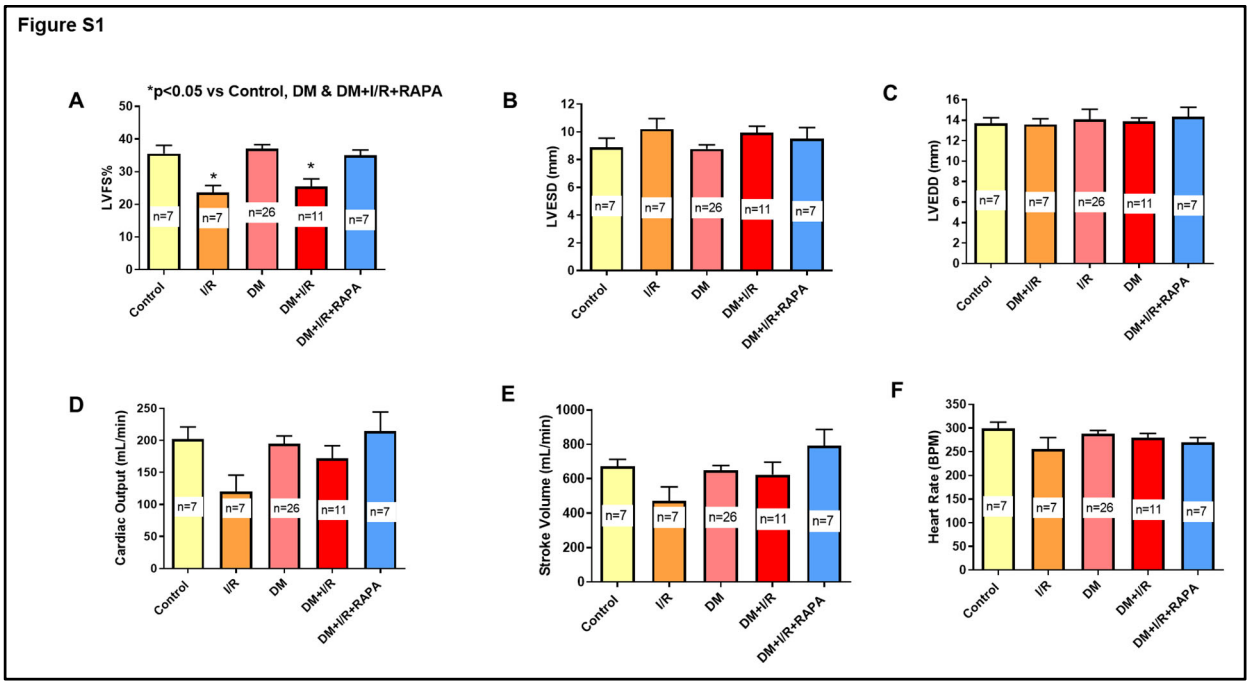
ID	Accession	Chromosome	Start	End	Strand
ocu-mir-191	MI0030998	chr9	16649737	16649828	-
ocu-mir-290	MI0030993				
ocu-mir-294	MI0030992				
ocu-mir-302a	MI0030989	chr15	36769946	36770014	+
ocu-mir-302b	MI0030987	chr15	36769627	36769706	+
ocu-mir-302c	MI0030988	chr15	36769770	36769837	+
ocu-mir-302d	MI0030990	chr15	36770109	36770176	+
ocu-mir-367	MI0030991	chr15	36770233	36770300	+
ocu-mir-498	MI0030995				
ocu-mir-512a	MI0030996				
ocu-mir-512b	MI0030997				
ocu-mir-520e	MI0030994				

B Sequence homology of miR-302a among human, rabbit, mouse and monkey (http://tcoffee.crg.cat)

Accession No	Species	miR Name	miR Sequence
			Seed Region
(MIMAT0000684)	Human	hsa-miR-302a	UAAGUGCUUCCAUGUUUUGGUGA
(MIMAT0036321)	Rabbit	ocu-miR-302a	UAAGUGCUUCCAUGUUUUGGUGA
(MIMAT0000380)	Mouse	mmu-miR-302a	UAAGUGCUUCCAUGUUUUGGUGA
(MIMAT0006259)	Monkey	mml-miR-302a	UAAGUGCUUCCAUGUUUUGGUGA
		cons	**********

http://www.mirbase.org/cgi-bin/query.pl?terms=Oryctolagus+cuniculus&submit=Search

Bioinformatics annotation of microRNAs in Rabbit. Related with Figure 4B and Figure S4.
(A) Details of 12 miRNAs in rabbits. (B) Multiple sequence alignment of miR-302a in human, rabbit, mouse and monkey. * indicates the presence of consensus sequence of miR-302a between human, rabbit, mouse and monkey.



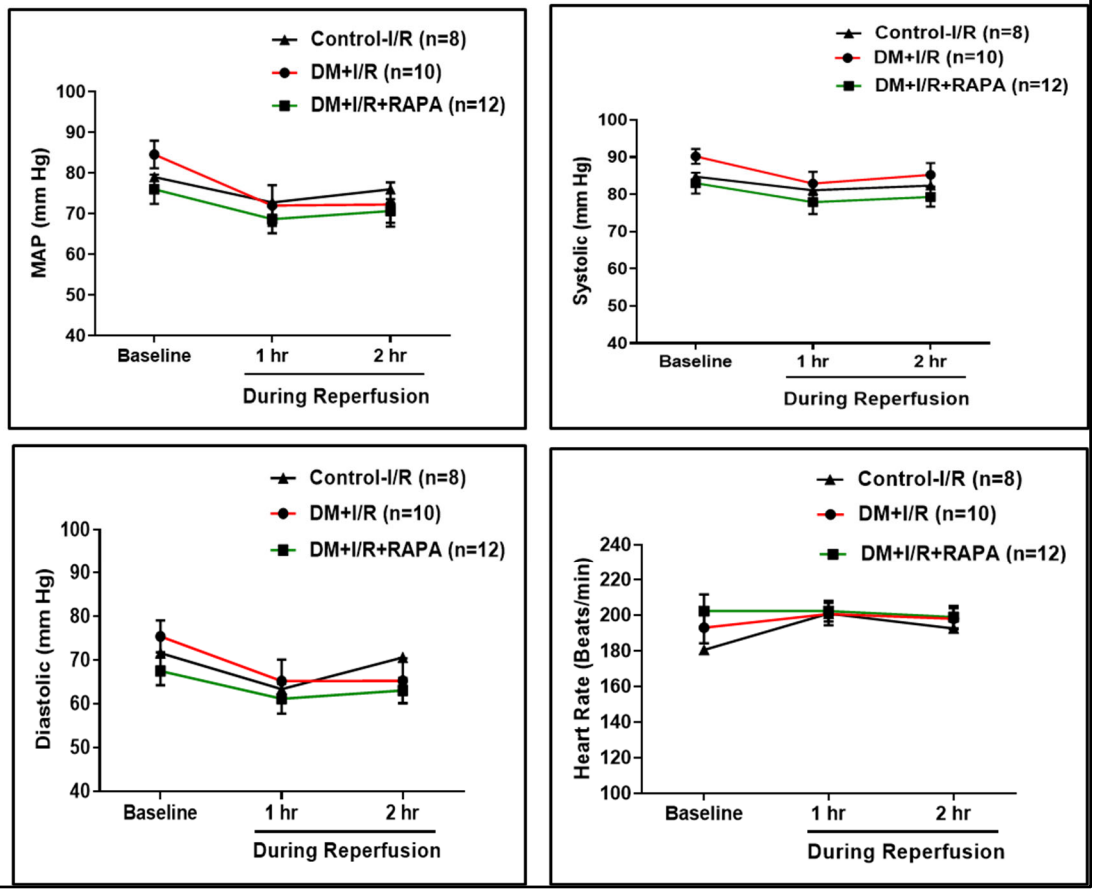
Cardiac function of control and diabetic (DM) rabbits before and after ischemia/reperfusion (I/R) injury with/without rapamycin (RAPA) treatment. Related to Figure 2.

Rabbits were subjected to 45 min conscious I and 72 hours of reperfusion. Diabetic rabbits were treated with RAPA at the onset of reperfusion (DM+I/R+RAPA).

(A) Percentage of left ventricular fractional shortening (LVFS, *p<0.05 vs Control, DM & DM+I/R+RAPA),
(B) LV end systolic diameter (LVESD),
(C) LV end diastolic diameter (LVEDD),
(D) Cardiac output,
(E) Stroke volume,
(F) Heart rate

Statistics: One-way ANOVA. Data are represented as mean ± SEM.

Figure S2



Hemodynamic measurements in control and diabetic rabbits after ischemia/reperfusion (I/R) injury with/without rapamycin (RAPA) treatment. Related to Figure 1B and Figure 2.

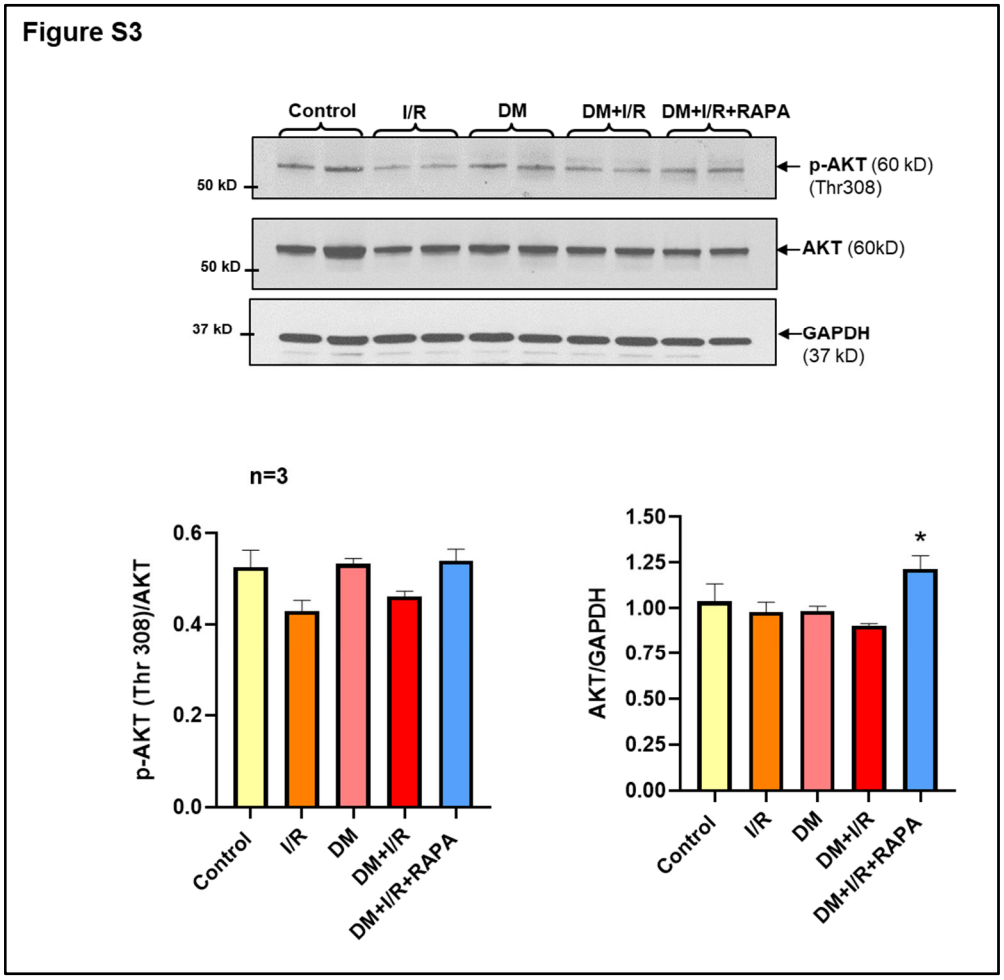
(A) Mean Arterial Pressure (MAP);

(B) Systolic Pressure (SBP);

(C) Diastolic Pressure (DBP);

(D) Heart rate (HR) in control (I/R) and diabetic rabbits after I/R (DM+I/R) and with RAPA (DM+I/R+RAPA). Acute administration of RAPA (0.25 mg/kg of BW; i.v.) was well tolerated by rabbits during the treatment procedure and had no significant influence on hemodynamic parameters.

Statistics: One-way ANOVA. Data are represented as mean ± SEM.

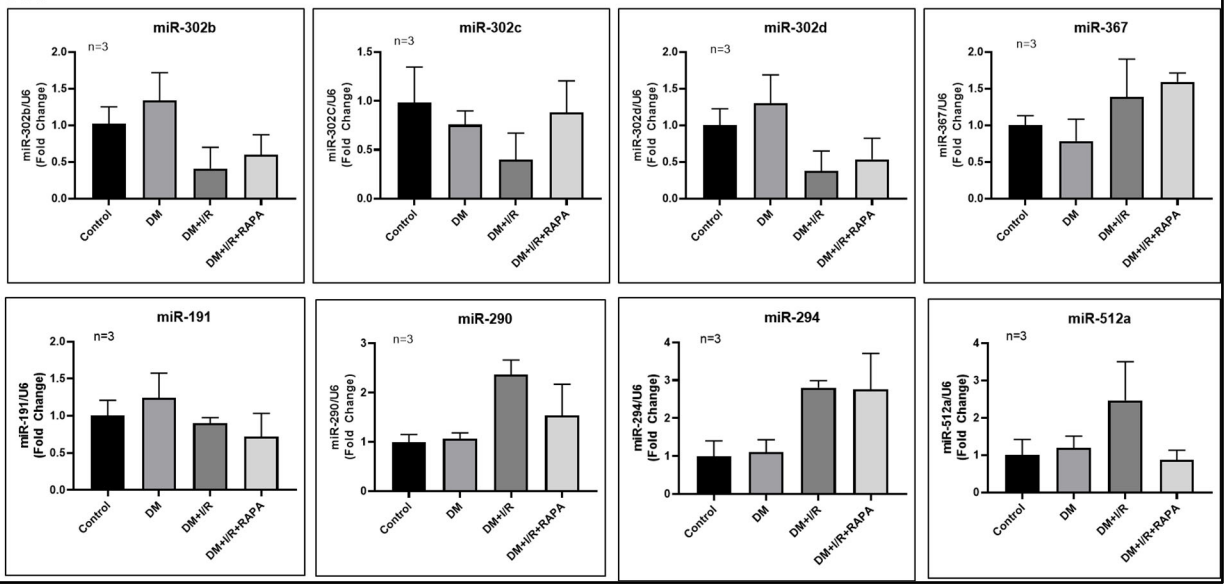


Phosphorylation of AKT at Thr308 in diabetic rabbits following ischemia/reperfusion (I/R) injury with/without rapamycin (RAPA) treatment. Related to Figure 4A.

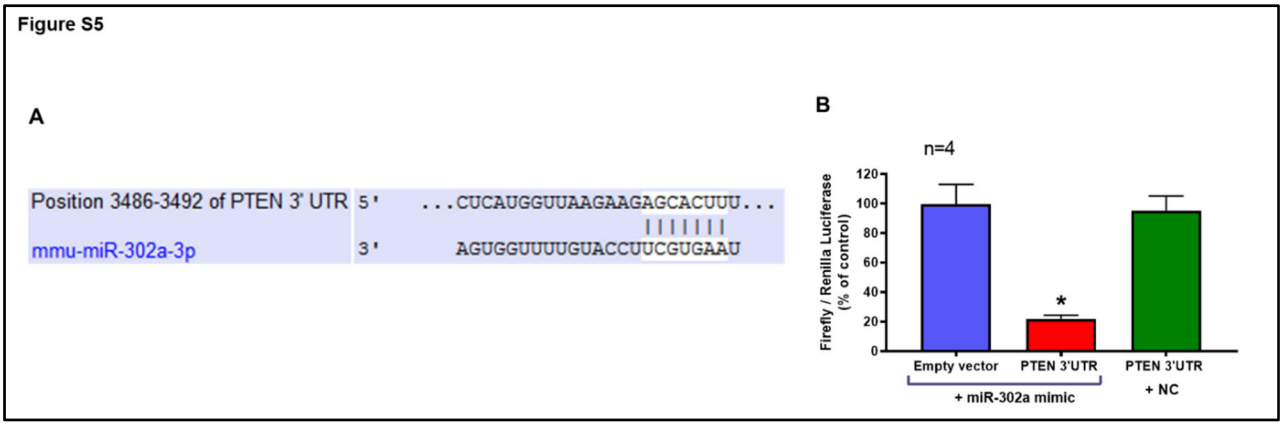
Upper panel shows the representative immunoblots and lower panel shows densitometry analysis of the ratios of phosphorylation of AKT to total AKT (p-AKT/AKT) and total AKT to GAPDH in control and diabetic rabbit hearts following I/R injury (n=3; *$p<0.05$ vs DM+I/R). Diabetic rabbits were treated with RAPA at the onset of reperfusion (DM+I/R+RAPA).

Statistics: One-way ANOVA. Data are represented as mean ± SEM.

Figure S4



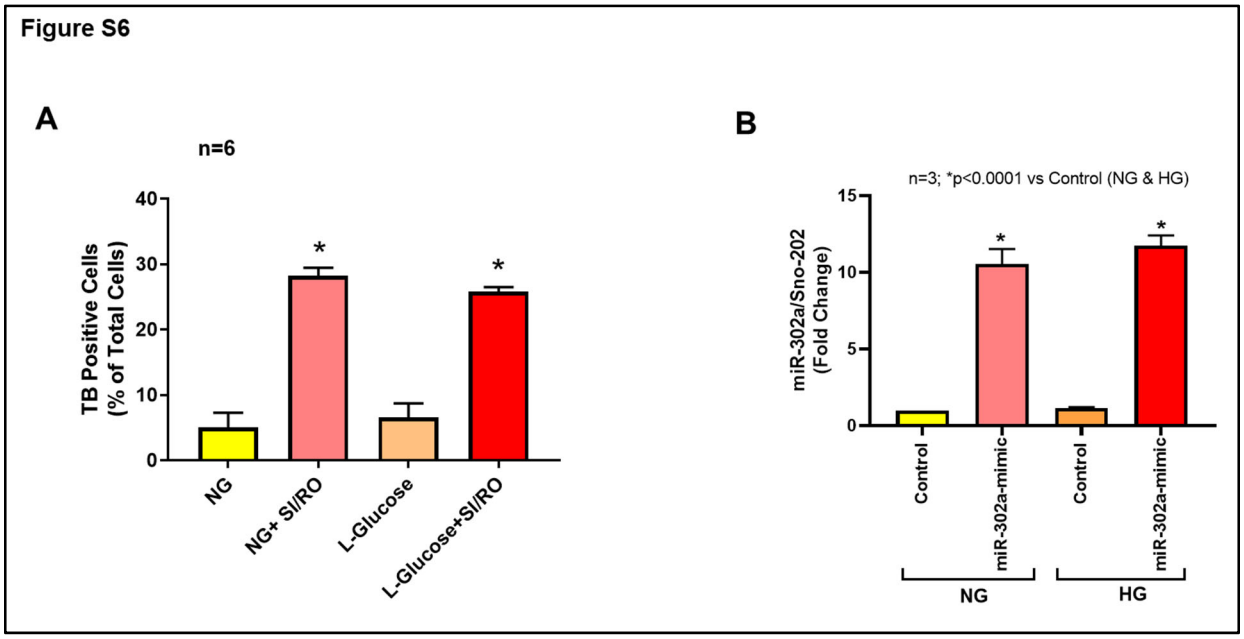
Expression profiles of miRNAs. Related to Figure 4B.
Expression profiles of miR-302b, miR-302c, miR-302d, miR-367, miR-191, miR-290, miR-294 and miR-512a in hearts of control and diabetic rabbits (DM) with/without rapamycin (RAPA) treatment during reperfusion.
Statistics: One-way ANOVA. Data are represented as mean ± SEM.



Interaction between miR-302a and 3'UTR of PTEN. Related to Figure 4C and D, 6D and 8C.
A. Prediction of two binding sites for mmu-miR-302a-3p in the 3′-UTR (untranslated region) of PTEN.

B. Dual-luciferase assays showed that the reduction of luciferase activity with the 3'-UTR of PTEN with mimic of miR-302a compared with the negative control (NC) of miR-302a binding site. n=4; *p<0.001 vs others.

Statistics: One-way ANOVA. Data are represented as mean ± SEM.



A. Effect of L-Glucose on necrosis of iPSC-CMs. Related to Figure 5, 6, 7 and 8.
iPSC-CMs were treated with L-glucose (25 mM) for 72 hours and subjected to SI/RO (simulated ischemia for 4 hours) and reoxygenation for 24 hours) injury under normal (NG) and high glucose (HG) conditions. n=6; *p<0.0001 vs NG & L-Glucose.
Statistics: One-way ANOVA. Data are represented as mean ± SEM.

B. miR-302a expression in iPSC-CMs after transfection with miR-302a mimic. Related to Figure 7 and 8. Time PCR quantitation of miR-302a in iPSC-CMs after 24 hours of transfection with miR-302a-mimic (20 nM) and 72 hours of normal (NG) and high glucose (HG) conditions. Sno-202 was used to normalize miRNA-302a expression. n=3; *p<0.0001 vs Control (NG & HG).
Statistics: One-way ANOVA. Data are represented as mean ± SEM.

Transparent Methods

Study Design

To elucidate the distinct signaling mechanisms involved in salutary effect of rapamycin (RAPA), we performed conscious myocardial ischemia/reperfusion (I/R) injury in preclinical diabetic rabbit and simulated ischemia/reoxygenation (SI/RO) injury in high-glucose (HG)-treated (human induced pluripotent stem cells-derived cardiomyocytes (hiPSC-CMs). Before induction of diabetes with alloxan, we ensured the similar average initial body weight and cardiac function in aged-matched rabbits. After alloxan treatment, body weight and glucose levels were monitored twice a day to confirm the induction of diabetes. Control and diabetic rabbits were randomized and subjected to conscious I/R injury with/without infusion of RAPA at the onset of reperfusion. After 72 hours of reperfusion, cardiac function was monitored. After sacrifice, myocardial infarct size, apoptosis, plasma troponin I, mTOR activity, expression of microRNAs (miRs) and PTEN were assessed. Typically, minimum four to six samples were used for *in vivo* experiments. The experimenters were blinded to group assignment and the outcome of the assessment. Data from six rabbits, which died within 14 days of alloxan treatment, were excluded from this study. Four rabbits were not included in I/R protocol because their blood glucose level were below 220 mg/dL. To examine the cause and effect relationship of miR-302a in regulating PTEN-AKT signaling, *in vitro* studies with three to six independent experiments were performed in HG-treated hiPSC-CMs with inhibition or overexpression of miR-302a followed by SI/RO.

Induction of diabetes in rabbit

New Zealand male rabbits (age: 3-4 months; body weight (BW): 2.8-3.0 kg; n=**90**) were purchased from Robinson Services Incorporated (RSI, NC, USA). All animal experiments were performed in accordance with USDA regulations and were approved by the Institutional Animal Care and Use Committee at the Virginia Commonwealth University. Diabetes in rabbits was induced as described by Wang *et al.*, 2010 (Wang et al., 2010) with modifications (**Figure 1A**). Briefly, alloxan monohydrate (125 mg/kg of BW, Sigma Aldrich, MO, USA) was administered via ear vein for 10 minutes in lightly sedated rabbits (**n=63**) with ketamine (35 mg/kg of BW), Xylazine (5 mg/kg of BW) and Atropine (5 mg/kg of BW). Blood glucose level was measured using Contour glucose meter (Bayer, NJ, USA) at 1, 2, 3 and 4 hours post-alloxan injection to prevent hypoglycemic shock. If blood sugar level dropped below 70 mg/dL, animals were supplemented with dextrose (10 ml of 10%, i.m.). Animals were provided with 20% glucose in drinking water for 3 days. Glucose levels were carefully monitored twice a day and animals were given insulin (1-2 U/kg of BW, Novalin-R, Nova Nordisk Pharmaceutical, NJ, USA) if glucose level exceeded 400-500 mg/dL. Body weights (BW) were also recorded weekly.

Conscious ischemia/reperfusion injury

After 4 weeks of alloxan treatment, animals with blood glucose consistently above 220 mg/dL were considered diabetic and included in the protocol for further experiments. Rabbits were randomized into five groups: Control (n=7), I/R (n=20), DM (n=10), DM with I/R (DM+I/R, n=23) and DM+I/R treated with RAPA (DM+I/R+RAPA, n=20). The rabbit model of conscious I/R has been described previously (Jones et al., 2015; Torrado et al., 2018). Briefly, after anesthetizing with sodium pentobarbital (35 mg/kg of BW; i.v.), the rabbit was intubated and connected to a ventilator (28-30 breaths/min). The heart was exposed through a left thoracotomy in the fourth intercostal space. After opening the pericardium, a 3-0 taper needled silk suture was passed beneath a major branch of the left coronary artery perpendicularly. A balloon occluder was placed on top of the coronary artery and secured with the 3-0 silk on the anterior LV wall and the chest wall was closed. Seven days after successful implantation of balloon occluder, the sedated rabbits were subjected to a 45-min conscious ischemia followed by 3 days of reperfusion by inflating/deflating the hydraulic balloon occluder. Ketoprofen (3.0 mg/kg, s.c.) was administered 2 h before and diazepam (4 mg/kg of BW; i.p.) was given 20 min before the onset of ischemia. Based on the treatment groups animals were infused with either RAPA (0.25 mg/kg of BW; i.v.) or DMSO (vehicle) 5 min before the onset of reperfusion via marginal ear vein catheter. Blood was collected in heparinized tube at baseline and at an interval of 1, 2, 4 and 24 hours after the initiation of reperfusion and centrifuged to separate plasma which was stored at -20^{o}C.

Assessment of Cardiac Function

Echocardiographic measurements were performed following a sedation protocol (inhaled isoflurane 2.5%) using a Vevo2100^{TM} (VisualSonics Inc., Toronto, Canada) at different time points, before (baseline) and after alloxan treatment, after implantation, and 3 days post I/R as per protocol to randomize the rabbits in DM, DM+I/R, DM+I/R+RAPA cohorts. Two operators, blinded to rabbit cohort allocation, performed repeated rounds of echocardiography to minimize inter- and intra-observer variations. Short and long parasternal views were obtained to measure cavity dimensions. The LVEF, left ventricular end-diastolic, end-systolic and stroke volumes were calculated by tracing the end- and epicardial boarder during contraction (Torrado et al., 2018). The obtained images were analyzed using Vevo LAB 3.2.0 software.

Measurement of hemodynamics

The animals were stabilized for at least 15 min and vital cardiac parameters including mean arterial pressure, systolic pressure, diastolic pressure and heart rate were monitored throughout the conscious I/R protocol via a transducer sensor probe secured to arterial line of rabbit ear connected to Blood Pressure Analyzer (BPA, Digi-Med, KY USA).

Infarct size measurement

After completion of I/R protocol, the rabbits were anesthetized with pentobarbital (50 mg/kg of BW; i.v.) and euthanized using potassium chloride via catheter connected to ear auricular artery. After excision, the heart was mounted on Langendorff apparatus and perfused with Krebs-Henseleit buffer. The coronary artery was tied and 5% solution of phthalo blue dye infused through the aortic root. The heart was cut into 5-6 transverse slices and stained with 1% triphenyltetrazolium chloride (TTC) and then fixed in 10% formalin and photographed (Torrado et al., 2018). The red region was marked as viable area, blue tissue as non-risk region and white area was considered as necrotic (**Figure 1C**). The infarct size was quantified using image J software (Bethesda, NIH, USA).

Evaluation of Apoptosis

The risk area of the LV was dissected, fixed in 10% formalin and 5µm thick sections were prepared after paraffin embedding. The sections were stained using a Terminal Deoxynucleotidyl Transferase dUTP Nick end Labeling (TUNEL) kit (BD Bioscience, San Jose, CA). The slides were then counter stained with troponin (mouse Troponin T antibody, Sigma-Aldrich, Missouri, USA) and anti-mouse IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 594 Conjugate, Cell Signaling, MA, USA), and fixed with DAPI anti-fade mount solution. The apoptotic cells (green nuclei) and total cells (DAPI-blue nuclei) were counted under a fluorescence microscope, and the data was plotted as the percentage of apoptotic cells to total cells.

Cardiac Troponin I measurement

Cardiac troponin I (cTnI) was measured in the plasma samples using Ultra-Sensitive Rabbit Cardiac Troponin-I ELISA kit (Life Diagnostics Inc, USA) (Jones et al., 2015; Torrado et al., 2018). Each assay was performed in duplicate in a blinded fashion.

Simulated Ischemia/Reoxygenation (SI/RO) in hiPSC-CMs

iCell Cardiomyocytes, human cardiomyocytes derived from induced pluripotent stem cells (hiPSC-CM, Cellular Dynamics International, CDI INC. Madison, WI) were treated with Maintenance Medium containing 10 mM Galactose in addition to glucose (NG:5 mM) or high glucose (HG:25 mM) for 72 hours (Rana et al., 2012). Subsequently, the cells were subjected to SI for 4 hours in a tri-gas incubator by adjusting 1-2% O_2 and 5% CO_2 at 37°C with an "ischemia buffer" (Das et al., 2006; Das et al., 2005). The cells were reoxygenated (RO) under normoxic conditions for 24 hours by replacing the ischemic buffer with normal Maintenance medium in addition to NG or HG.

A subset of cells were treated with RAPA (100 nM) during RO under both NG and HG conditions. Cell death was assessed by Trypan blue exclusion assay and apoptosis by TUNEL staining (Das et al., 2006; Das et al., 2005).

Bioinformatic analysis of Rabbit miRNA

Based on the annotation data obtained from miRBASE (http://www.mirbase.org), 12 potential miRNAs were predicted to express in the rabbit species **(Table S2)**. We analyzed the expression profile of 9 microRNAs, which showed sequence similarity to human isoforms by real time PCR. Moreover, target prediction analysis using TargetScan (Garcia et al., 2011; Lewis et al., 2005) identified the potential binding site for miR-302a-3p on the 3'UTR of PTEN mRNA **(Figure S5)**. Published literature (Poliseno et al., 2010) also provided further evidence for miR-302a and PTEN interaction.

PTEN 3'UTR Luciferase Assay

The specificity of miR-302a binding to 3'-UTR of PTEN was confirmed by Dual luciferase reporter assay. The fragment of PTEN 3'-UTR (3001-3997) which includes the binding region of miR-302a was amplified from mouse genomic DNA and cloned into pmirGLO Dual-Luciferase miRNA target expression Vector (Promega Corp., WI, USA) using Sac1 and Xba1 restriction sites. H9C2 cells (ATCC, VA, USA) were transfected with pmiRGLO vector containing PTEN 3'UTR binding sequences for miR-302a or empty vector along with either miR-302a mimic (miR-302a-3p-5'uaagugcuuccauguuuugguga-3') or miR mimic negative control (Applied Biological Materials Inc., BC, Canada) using Lipofectamine 2000 (Invitrogen, NY, USA). The intensity of firefly luciferase corresponding to miR-302a activity was analyzed using Dual luciferase assay system (Promega Corp., WI, USA). Renilla luminescence served as internal control for transfection efficiency and normalization. The data was presented as percentage of Firefly to Renilla luciferase ratio normalized to empty vector control (**Figure S5B**). The following primers were used to amplify the PTEN-3'UTR from mouse genomic DNA: PTEN-Sac1-FP-5'-ACGAGCTCCCATCTCCTATGTAATC-3'and PTEN-Xba1-RP-5'-TCTCTAGAGAGTGAAACTGATGAGGTAT-3'.

LNA based miRNA-302a inhibition and miRNA-302a overexpression

LNA™-enhanced hsa-miR-302a-3p inhibitor **(**miRCURY-LNA-Power Inhibitor, 5'-fluorescein labeled; $^{5'}$-CACCAAAACATGGAAGCACTT-$^{3'}$/36-FAM/) was purchased from Exiqon Company (Woburn, MA, USA). miRCURY-LNA-miR-power inhibitor contains phosphorothioate bonds which enhances the transfection efficiency of iCell Cardiomyocyte. miCURY-LNA^{TM}-miR inhibitor control ($^{5'}$-TAACACGTCTATACGCCCA-$^{3'}$) was used as a negative control (scramble). miR-302a mimic (hsa-miR-302a-3p mimic) was purchased from Applied Biological Materials Inc (Richmond, BC, Canada).

Transfection of hiPSC-CMs

hiPSC-CM cells were transfected with hsa-miR 302a-3p inhibitor (20 nM) or miR-control inhibitor or miR-302a mimic (20 nM) using ViaFect^{TM} Transfection Reagent (Promega Corp. Madison, WI). After 48 hours, cells were subjected to high glucose (HG) conditions for 72 hours and SI/RO protocol.

Western blot analysis

Total soluble protein was extracted from the frozen LV tissues/hiPSC-CMs with lysis buffer (Cell Signaling, MA, USA). Protein samples (50 µg) were resolved by SDS-PAGE, transferred onto a nitrocellulose membrane. Membranes were incubated overnight with mouse monoclonal antibody specific for PTEN, p-AKT (S^{473}), p-AKT (Thr308), AKT, p-S6, S6, Bcl-2, Bax and GAPDH (Cell Signaling, MA, USA). The blots were then incubated for 1-hour with anti-mouse secondary horseradish peroxidase-conjugated antibody (GE healthcare, PA, USA) and developed using

Western Lightning Plus–ECL substrate (PerkinElmer, MA, USA). The densitometry analysis to quantitate the intensity of the protein band was performed using Image J software (NIH, Bethesda, MD).

Lipid Peroxidation Assay

Lipid peroxidation was measured by formation of malondialdehyde (MDA) in heart tissues using an assay kit (BioVision, CA, USA) according to the manufacturer's protocol (Das et al., 2014).

RNA isolation and miRNA expression

Total RNA was isolated from frozen LV tissue/hiPSC-CMs using miRNeasy mini kit (QIAGEN Sciences, MD, USA). Concentration and the purity of the isolated RNA was verified using Nanodrop ND-1000 spectrophotometer (Agilent technologies, CA, USA). Total RNA (10 ng) was subjected to reverse transcription reaction with miRNA-specific RT-primer using microRNA reverse transcription kit (Applied Biosystems, CA, USA). TaqMan™ miRNA assay probe (Occ-miR-302a-UAAGUGCUUCCAUGUUUUGGUGA) was performed (Applied Biosystems, CA, USA) to determine the expression level of miR-302a and normalized using U6 or Sno-202 small RNA. All other miRNAs (miR-191, miR-290, miR-294, miR-512a, miR-302b, c,d, miR-367) were quantified using TaqMan™miRNA Assay from Applied Biosystems. To quantify the mRNA of PTEN, total RNA (2 µg) were reverse transcribed using high capacity cDNA synthesis kit (Applied Biosystems). cDNA was subjected to real-time-PCR using TaqMan™ mRNA Assay for PTEN quantification and normalized with GAPDH using Roche Light cycler 480 II (Roche Applied Science, IN, USA).

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 8 (GraphPad Software Inc. La Jolla, CA). The data are presented as mean±SE for each treatment group, along with unadjusted 2-tailed p values <0.05 were considered statistically significant. One-way ANOVA + Bonferroni post-hoc test was used for unpaired data to compare 2 and 3 groups, respectively. Two-way analysis of variance (ANOVA) with repeated measures + Bonferroni post hoc test were used to compare pre-intervention and post-intervention variables when appropriate.

Ethics Statement

All animal experiments were performed in accordance with USDA regulations and were approved by the Institutional Animal Care and Use Committee at the Virginia Commonwealth University.

Supplemental References

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