Regulation of Cardiac Expression of the Diabetic Marker MicroRNA miR-29

Nicholas Arnold¹,³, Purushotham Reddy Koppula¹,³, Rukhsana Gul¹,³,⁴, Christian Luck¹,³, Lakshmi Pulakat¹,²,³*

¹Department of Medicine, University of Missouri, Columbia, Missouri, United States of America, ²Department of Nutrition and Exercise Physiology, University of Missouri, Columbia, Missouri, United States of America, ³Harry S Truman Memorial Veterans Affairs Hospital, Columbia, Missouri, United States of America, ⁴Obesity Research Center, College of Medicine, King Saud University, Riyadh, Saudi Arabia

Abstract

Diabetes mellitus (DM) is an independent risk factor for heart disease and its underlying mechanisms are unclear. Increased expression of diabetic marker miR-29 family miRNAs (miR-29a, b and c) that suppress the pro-survival protein Myeloid Cell Leukemia 1 (MCL-1) is reported in pancreatic β-cells in Type 1 DM. Whether an up-regulation of miR-29 family miRNAs and suppression of MCL-1 (dysregulation of miR-29-MCL-1 axis) occurs in diabetic heart is not known. This study tested the hypothesis that insulin regulates cardiac miR-29-MCL-1 axis and its dysregulation correlates with DM progression. In vitro studies with mouse cardiomyocyte HL-1 cells showed that insulin suppressed the expression of miR-29a, b and c and increased MCL-1 mRNA. Conversely, Rapamycin (Rap), a drug implicated in the new onset DM, increased the expression of miR-29a, b and c and suppressed MCL-1 and this effect was reversed by transfection with miR-29 inhibitors. Rap inhibited mammalian target of rapamycin complex 1 (mTORC1) signaling in HL-1 cells. Moreover, inhibition of either mTORC1 substrate S6K1 by PF-4708671, or elf4e-induced translation by 4E1RCat suppressed MCL-1. We used Zucker diabetic fatty (ZDF) rat, a rodent model for DM, to test whether dysregulation of cardiac miR-29-MCL-1 axis correlates with DM progression. 11-week old ZDF rats exhibited significantly increased body weight, plasma glucose, insulin, cholesterol, triglycerides, body fat, heart weight, and decreased lean muscle mass compared to age-matched lean rats. Rap treatment (1.2 mg/kg/day, from 9-weeks to 15-weeks) significantly reduced plasma insulin, body weight and heart weight, and severely dysregulated cardiac miR-29-MCL1 axis in ZDF rats. Importantly, dysregulation of cardiac miR-29-MCL-1 axis in ZDF rat heart correlated with cardiac structural damage (disorganization or loss of myofibril bundles). We conclude that insulin and mTORC1 regulate cardiac miR-29-MCL-1 axis and its dysregulation caused by reduced insulin and mTORC1 inhibition increases the vulnerability of a diabetic heart to structural damage.

Introduction

Several epidemiological studies including the Framingham Study, UK Prospective Diabetes Study (UKPDS), Cardiovascular Health Study, and the Euro Heart Failure Surveys provide strong evidence for the fact that diabetes mellitus (DM) is an independent predictor for heart disease [1–4]. The fact that the adults with diabetes have heart disease death rates about 2–4 times higher than adults without diabetes strongly suggests that the compensated heart in DM is very vulnerable to sudden malfunction resulting in death. In addition to the well-studied left ventricular (LV) dysfunction in DM, recent studies have highlighted the involvement of right ventricular (RV) dysfunction in diabetic heart disease [5,6]. However, mechanisms underlying diabetic cardiomyopathy are still elusive. Identifying DM-specific molecular changes that increase the vulnerability of cardiac myofibrils to structural damage is of high utility in developing new therapeutics and regimens to control heart disease in diabetic individuals.

In this context, the diabetic marker microRNA miR-29 family that plays a role in increasing cell death is particularly noteworthy. The miR-29 family consists of miR-29a, b (b1 and b2) and c that are located on two different chromosomes (chromosomes 4 and 13 in rat, 1 and 6 in mouse and 1and 7 in human) [7]. Quantitative trait loci (QTLs) associated with rat miR-29a and b highlight potential involvement of miR-29a and b in cardiovascular diseases (Fig. 1A). miR-29a was identified as one of the miRs that was up-regulated in the serum of children with Type 1 DM (T1DM) [8]. In diabetic mice, an increase in miR-29c was associated with podocyte cell death that underlies diabetic nephropathy. Additionally, knock-down of miR-29c suppressed high glucose induced apoptosis of podocytes and improved kidney function [9]. Increase in miR-29b leads to the development of aortic aneurisms [10]. Suppression of miR-29 by anti-miR-29 oligomers protects against myocardial ischemia-reperfusion injury, abdominal aortic aneurism and diabetic nephropathy [9–13]. miR-29 is also one of the several miRNAs associated with inflammatory microvesicles [14]. In non-obese diabetic (NOD) mice, up-regulation of miR-29a, b

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* Email: pulakatl@health.missouri.edu
insulin is an activator of the nutrient sensor kinase mammalian target of rapamycin complex 1 (mTORC1), we further posited that mTORC1-signaling mediates insulin’s effects on miR-29-MCL-1 axis. To evaluate the effect of insulin and mTORC1 on miR-29-MCL-1 axis in cardiomyocytes, we used mouse atrial cardiomyocyte cell line HL-1 [25] and tested the effects of Rap treatment on miR-29-MCL-1 axis regulation. To determine how DM progression (natural or advanced by Rap treatment) caused dysregulation of cardiac miR-29-MCL-1 axis and promoted cardiomyocyte disorganization, we used male ZDF rats, a well-established rodent model for advanced DM [5,6,26,27] and evaluated the correlation between regulation of miR-29-MCL-1 axis and disorganization of myofibril bundles in cardiac right ventricle.

Methods

Cell culture and treatments

The cardiac muscle cell line HL-1 (a generous gift from Dr. William Claycomb, - Louisiana State University Medical Center) is an atrial cardiomyocyte tumor lineage originally derived from female AT-1 mouse [25]. Cells were cultured in a 37°C incubator in the presence of 5% CO₂ in complete Claycomb medium using flasks pre-coated with 12.5 μg/ml bovine fibronectin (Sigma) in 0.02% gelatin solution (Sigma, St Louis, MO) as described previously [25,28]. Confluent cells were washed with 1X phosphate buffered saline (PBS), and incubated in serum-free Claycomb medium prior to treatments with bovine insulin (100 nM;12 hr), rapamycin (10 nM:12 hr), p70S6K1 inhibitor PF-4708671(2 μM;12 hr), or 4E1RCat, an inhibitor of cap-dependent translation (5 μM;12 hr). Insulin was purchased from Sigma-Aldrich Inc., rapamycin from Cell signaling Technology, and PF-4708671 and 4E1RCat were from Tocris Bioscience. At the end of treatments with different agents, medium was removed, cells were rapidly cooled with ice-cold PBS, collected using cell scrapers followed by centrifugation at 3500 rmps for 5 minutes at 4°C, and cell pellets were flash frozen using liquid nitrogen. Cell pellets were stored in a -80°C ultra-freezer until further processing. All treatments were performed at least in triplicate.

Animals, Rap treatment, and Body composition

All animal procedures used in this study were approved prior to the beginning of these studies by the Harry S. Truman Veterans Memorial Hospital (HSTVMH) Subcommittee for Animal Safety and University of Missouri IACUC. All animals were cared for in accordance with the Guidelines for the Care and Use of...
Laboratory Animals (National Institutes of Health publication 85-23). Nine-week old male ZL and ZDF rats (24 animals) purchased from Charles River Laboratories were used in this study. Animals were housed at the HSTVMH animal housing facility under standard laboratory conditions (room temperature: 21–22 °C; light and dark cycles: 12 h). Rats were maintained on ad libitum food and water. Rapamycin pellets designed to deliver Rap at a concentration of 1.2 mg/kg/day for 21 days (from Innovative Research of America, Inc, Sarasota, FL) or placebo pellets were placed surgically under the skin behind the shoulder blades under brief isoflurane anesthesia and this procedure was repeated to achieve a 6-week treatment. Body composition in ZL and ZDF rats was determined using the EchoMRI 4in1/1100. EchoMRI is the preferred non-invasive method to measure body composition because it is a rapid measurement that can be performed on live, un-anaesthetized animals [29]. The EchoMRI 4in1/1100, is a QMR system that measures lean mass, fat mass, total water, and free water. The rats were placed in an adjustable plastic cylinder to restrict movement. The cylinder (2.75 inches in diameter) has openings on either end to allow the animals to breathe freely. The cylinder was inserted into the EchoMRI for a reading that lasted for 85 seconds.

Blood and tissue collection, plasma analysis, histopathology

Animals were fasted for 6 hours before blood collection. Blood was collected biweekly from the saphenous vein. Blood was also collected by cardiac puncture at the time of sacrifice according to IACUC approved procedure. Rats were acclimated for two weeks to reduce stress before starting blood draw. The back of the chosen leg was shaved to make the saphenous vein visible. A compression point at the base of the leg was used to make the saphenous vein hulge out. A 20 G needle was used to puncture the vein to collect the blood. Prior to euthanasia, rats were given intraperitoneal injection with 50 mg/kg of sodium pentobarbital to properly anesthetize them. Animals were euthanized by opening of the chest cavity, removal of 6–10 mls of blood from the heart, followed by removal of the heart. Plasma analysis was performed by Comparative Clinical Pathology Services at Columbia. Plasma levels of cholesterol and triglycerides were measured using commercially available assays (Beckman-Coulter, Brea, CA) on an automated clinical chemistry instrument (AU680, Beckman-Coulter, Brea, CA). Glucose and insulin were measured by an automated hexokinase G-6-PDH assay and an ELISA kit specific for rat insulin, respectively.

Heart tissue from right ventricle (RV) was used in this study for RNA, protein and histopathology analysis. Tissues for RNA isolation and protein analysis were rapidly flash frozen in liquid nitrogen in aluminum foil packets that were pre-cooled on dry-ice. Tissues were fixed in 10% neutral buffered formalin (NBF), embedded into paraffin blocks, sections were cut at 4 μm thickness, and were stained with haematoxylin and cosin (H&E), Masson’s Trichrome Stain (MTS), and anti-α-actin antibody at Research Animal Diagnostic Laboratory (RADIL), Columbia, Missouri. The stained sections were scanned using the Aperio CS Slide Scanner by WSI Analytics Lab, Department of Pathology and Anatomical Sciences, University of Missouri, Columbia.

RNA isolation and quantitative real-time RT-PCR

Isolation of mRNA and miRNA from frozen HL-1 cell pellets and RV tissues was performed using mirVana miRNA isolation kit (Ambion) following the manufacturer’s protocol. The mRNA and miRNA were quantified using NanoDrop (Thermo Scientific) and stored at −80 °C until further processing. c-DNA synthesis for mRNA and miRNA was carried out using Omniscript RT kit from Promega (Madison, WI) and Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems Life Technologies) respectively. Taqman microRNA assay primers for miR-29a, b and c and snRNA (Taqman microRNA Assays) and mouse and rat MCL-1 and 18S RNA primers (Gene Expression Assays) from Applied Biosystems Life Technologies were used in these experiments. qRT-PCR was carried out using cDNA generated from these RNA samples as templates. Experiments were performed in triplicates for each biological sample with Taqman Fast Universal PCR Master Mix 2X, (Applied Biosystems Life Technologies). snRNA and 18S RNA were used as internal controls for miRNA and mRNA respectively. qRT-PCR was performed using the Applied biosystems 7500 Fast PCR system. Relative quantification (RQ) values were obtained by determining ΔCt values followed by determining ΔΔCt values and then RQ values via the equation 2−ΔΔCt.

Immunoblotting

Frozen HL-1 cell pellets from various treatments were lysed using ice-cold nonidet lysis buffer [30] supplemented with okadaic acid (0.1 μM) and Na2VO3 (0.25 mM) to prevent Serine/Threonine and Tyrosine phosphatases. Cell debris was removed by centrifugation and protein in the supernatant was estimated by BCA method (Pierce BCA protein assay kit). Samples were normalized, and bsates corresponding to 60 μg of protein were subjected to SDS-PAGE analysis. Separated proteins were transferred to PVDF membrane (Millipore) by Western blotting. After blocking with 5% BSA, PVDF membranes were probed with primary antibodies for S6K1, phospho-S6K1(1pS6K1: pThr389), RPS6, phospho-RPS6(pRPS6: pSer235/236), 4E-BP, phospho-4E-BP(pE-BP: pThr37/Thr46) and MCL-1 in 5% BSA in TBST overnight (1:1000 dilution of each antibody; all antibodies from Cell Signaling Technology). Blots were then washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies (1:30,000 dilution of each antibody) for 1 hr at room temperature. Binding of the antibodies was detected by Chemiluminescence (Supersignal west femto maximum sensitivity substrate kit; Thermo Scientific), and images were captured using a Bio-Rad ChemiDoc XRS image-analysis system. All experiments were done at least in triplicates. Quantitation of phosphorylated protein band density, normalized to the density of total protein for each sample, was performed using Quantity One software (Bio-Rad Laboratories Inc. Berkeley, CA). Data are reported as the normalized protein band density in arbitrary units.

Immunofluorescence

HI-1 cells were grown on cover slips pre-coated with 12.5 μg/ml bovine fibronectin in 0.02% gelatin solution. Transfection was performed using siPORT Amine (Applied Biosystems) according to manufacturer’s protocol. 20 nM of miR-29 inhibitor cocktail (mirVana miRNA inhibitors for miR-29a, b and c) or 20 nM Allstars negative siRNA (Qiagen) was used for transfection. After 8 hours, cells were subjected to Rap treatment (10 nM) overnight. Coverslips were washed with PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized with 1% Triton-X, washed with PBS-T (1 mL, TWEEN-20/L), and blocked with background sniper (Biocare Medical). Anti-MCL-1 antibody (Cell Signaling Technology) (1:50 dilution) was added in fluorescent AB diluent (Biocare Medical) and incubated overnight at 4 °C. After repeated washing with PBS-T, coverslips were incubated with Alexa Fluor 488 goat anti-rabbit (Invitrogen Inc.) (1:200 dilution) for 1 hr at room temperature. After washing with PBS-T, coverslips were mounted on slides with Fluoroshield with 4′,6-
mTORC1-mediated phosphorylation of 4E-BP causes phosphorylated form binds to eIF4E and represses translation [34]. The 4E-BP in its hypophosphorylated state was achieved by incubation with PF-4708671 (2 mM) in HL-1 cardiomyocytes. S6K1 suppression of suppressing signaling by each of the mTORC1 substrates on cardiomyocyte signaling. Since increased expression of different members of miR-29 family is associated with DM, we tested the effects of insulin that attenuates the progression of DM, and rapamycin (Rap) that promotes the progression of DM, on the expression of miR-29 family miRNAs in HL-1 cells. In HL-1 cardiomyocytes, we determined whether insulin treatment (100 nM: 12 hr) induced phosphorylation of mTORC1 substrates. Though insulin treatment could induce phosphorylation of S6K1 rapidly, in this study we chose a 12 hr treatment to be consistent with the treatment time used for determining the changes in miR-29 and an MCL-1 mRNA expression in response to insulin in HL-1 cells. The S6K1 is phosphorylated at Thr389 by mTOR [32]. The phosphorylated S6K1 (pS6K1) activates Ribosomal protein S6 (pRPS6) via phosphorylation of five evolutionarily conserved residues of RPS6, Ser235, Ser236, Ser240, Ser244 and Ser247. pRPS6 is implicated in increasing translation and cell size [32,33]. The mTORC1 also phosphorylates 4E-BP at Thr47/Tyr46. Western blot analysis of the lysates from HL-1 cells treated with insulin confirmed that insulin induced phosphorylation of mTORC1 substrates and their down-stream targets (S6K1 at Thr389; RPS6 at Ser235/236) and 4E-BP at Thr384/46 in these mouse cardiomyocytes (Fig. 2C). Conversely, Rap treatment (10 nM: 12 hr) of HL-1 cells suppressed this effect (Fig. 2C).

Since insulin improved and Rap suppressed MCL-1 expression in HL-1 cardiomyocytes, we tested how the exposure of cells to insulin (100 nM: 12 hr) and Rap (10 nM: 12 hr) simultaneously modulates MCL-1 protein levels in these cells. Rap suppressed MCL-1 protein levels significantly (Fig. 2C). However, Rap-mediated suppression of MCL-1 was partly reversed by the presence of insulin (Fig. 2C). This observation suggested that insulin has a protective effect on MCL-1 protein expression.

Since MCL-1 is a target of miR-29 family miRNAs, and Rap treatment increases miR-29 expression, we investigated whether a miR-29 inhibitor cocktail (inhibitors of miR-29a, b and c) would improve MCL-1 expression in Rap treated HL-1 cells. HL-1 cells were transfected with either Allstars negative control siRNA or miR-29 inhibitor cocktail and after 8 hours of transfection subjected to treatment with Rap (10 nM) overnight. Immunofluorescence analysis using anti-MCL-1 antibody showed that Rap treatment substantially suppressed MCL-1 expression in HL-1 cells transfected with Allstars negative control siRNA, but not in HL-1 cells transfected with miR-29 inhibitor cocktail (Fig. 2D). This observation implied that miR-29 family miRNAs regulate MCL-1 expression in HL-1 cardiomyocytes.

**General characteristics and the status of cardiac miR-29-MCL-1 axis of 11-week old ZL and ZDF rats**

The ZDF rats are hyperphagic due to a leptin receptor mutation and insulinopenic due to a pancreatic dysfunction [26,27]. ZDF rats become hyperglycemic by the age of 6 weeks. To verify the general characteristics of ZL and ZDF rats used in this study we determined fasting plasma glucose, insulin, cholesterol and triglycerides at 11-weeks of age. As shown in Fig. 3 (A-D), ZDF rats exhibited significant increases in fasting glucose, insulin, cholesterol and triglycerides at 11-weeks compared to age-matched ZL rats. EchoMRI analysis of the body composition showed that the ZDF rats had substantial increases in total body fat mass and body weight, and a significantly decreased lean muscle mass (Fig. 3E-G). Thus, the ZDF rats used in this study exhibited hyperinsulinemia, hyperglycemia, hypercholesterolemia and muscle loss, characteristic of DM. Heart tissues of 11-week old ZDF rats showed a significant increase in their weight after
adj. to tibia length (3H) indicating cardiac hypertrophy compared to age-matched ZL rats. Since ZDF rats had ~14 fold higher plasma insulin compared to ZL rats (Fig. 3B), at 11-weeks they had compensative hyperinsulinemia. This is consistent with previous reports [26]. qRT-PCR analysis of cardiac miRNA showed that there was a moderate, but statistically significant increase in 29a and b, and a small, non-significant increase in 29c (Fig. 3I). qRT-PCR analysis of cardiac miRNA showed that ZDF rats exhibited about 45% decrease in MCL-1 expression compared to ZL rats (Fig. 3J). These data suggest that cardiac miR-29-MCL-1 axis is mildly dysregulated in 11-week old ZDF rats that suffer from DM.

Figure 2. INS and mTORC1 regulate cardioprotective MCL-1 mRNA expression in HL-1 cardiomyocytes. A). MCL-1 expression is up-regulated by treatment with INS (100 nM, 12 h) as determined by qRT-PCR. B) MCL-1 is suppressed by inhibitors of signaling induced by mTORC1 and its substrates p70 s6K1 and 4E-BP. qRT-PCR data showed that MCL1 mRNA levels in HL-1 cardiomyocytes were significantly suppressed in response to treatment with Rap (mTORC1 inhibitor; 10 nM; 12 hrs), or PF470867 (p70 s6K1specific inhibitor; 20 nM; 12 hrs) or 4E1RCat (a novel suppressor of elf4E-induced translation; 5 μM; 12 hrs). Treatments were performed at least four times for INS and each of the mTORC1 inhibitors. qRT-PCR for each biological sample was performed in triplicates. Comparative expression levels (RQ values) are expressed relative to untreated (Con) HL-1 cells. Values are means ± SEM. *p<0.01 for Con vs. INS, and Con vs. Rap, PF4708671 or 4E1RCat, C). Autoradiograms show in HL-1 cardiomyocytes levels of pThr389S6k1 (pS6K1), pSer235/236RPS6 (pRPS6), and pThr37/Thr464E-BP (p4E-BP) were elevated in response to INS (100 nM, 12 hr) treatment and were suppressed in response to Rap (10 nM, 12 Hr with or without INS). Graphs show results of densitometric analysis of the intensity of phosphorylated protein bands after adjusting to the intensity of total protein bands (tS6K1, tRPS6, t4E-BP) and to the intensity of the β-actin that was used as an internal control. N = 4 *p<0.01 for Con vs. INS, INS vs. INS+Rap, and INS vs. Rap. Western blotting also showed suppression of MCL-1 via Rap treatment in HL-1 cardiomyocytes (N = 4, *p<0.0002). There was no significant difference in the MCL-1 levels between control and HL-1 cells treated with INS+Rap (N = 4). However, there was significant difference in the MCL-1 levels between Rap treated and INS+Rap treated HL-1 cells (N = 4, **p<0.02). D) Immunofluorescence staining with anti-MCL-1 antibody and nuclear stain DAPI in HL-1 cells transfected with either (a) Allstars negative siRNA and (b) Allstars negative siRNA and treated with Rap (10 nM), or (c) miR-29 inhibitor cocktail (mirVana miRNA inhibitors for miR-29a, b and c) and treated with Rap (10 nM). Top panel shows DAPI staining. Bottom panel shows anti-MCL-1 antibody staining. Anti-MCL-1 antibody staining was substantially low in Rap treated HL-1 cells transfected with Allstars negative siRNA (b) compared to that in HL-1 cells transfected with Allstars negative siRNA without Rap treatment. Transfection of HL-1 cells with miR-29 inhibitor cocktail reversed Rap-mediated suppression of MCL-1 expression. doi:10.1371/journal.pone.0103284.g002

miR-29-MCL-1 Axis in Diabetic Heart Disease

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Effects of Rap-treatment of ZDF rats on general characteristics and the status of cardiac miR-29-MCL-1 axis of 11-week old ZL and ZDF rats.

Since Rap-treatment increased miR-29 levels and suppressed MCL-1 mRNA levels in mouse HL-1 cardiomycocytes, we tested whether Rap-treatment would increase cardiac miR-29 family miRNAs and suppress cardiac MCL-1 mRNA even further in young ZDF rats. Nine-week old male ZDF rats were subjected to Rap treatment (1.2 mg/kg/day) for 6 weeks (until they were 15-weeks old) by implanting Rap pellets subcutaneously. Diabetes in ZDF rats is known to progress from a stage of severe hyperinsulinemia to progressive insulin loss and finally to insulinopenia by the time they are 24 weeks of age [26]. As noted in Fig. 3B, the 11-week old ZDF rats were at a stage of severe hyperinsulinemia (11-week ZDF rat plasma insulin levels: 2587.5±217 pmol/l versus 11-week ZL rat plasma insulin levels: 172.5±48 pmol/l). 15-week old control ZDF rats had a 7-fold reduction in their plasma insulin (Fig. 4A; insulin levels: 337.9±40 pmol/l) compared to 11 week old ZDF rats. Thus 15-week old ZDF rats were at a stage of progressive loss of insulin indicating advancement of DM as described previously [26]. Nevertheless, 15-week old ZDF rats were still significantly hyperinsulinemic. Interestingly, in Rap treated ZDF rats, plasma levels of insulin were even more decreased (Fig. 4A; Rap-treated ZDF rat insulin levels: 157.4±27 pmol/l). Therefore, 15-week old Rap-treated ZDF rats had a 2 fold reduction in their fasting insulin levels compared to age-matched control ZDF rats.

Though Rap treatment decreased fasting plasma glucose levels, the Rap-treated ZDF rats still had very high fasting glucose levels (Fig. 4B; fasting glucose in Rap-treated rat: 22.4±0.7 nmole/l) suggesting that they were severely hyperglycemic. Thus Rap-treated ZDF rats were at a more advanced stage of DM compared to age-matched control rats that did not receive Rap since they exhibited severe hyperglycemia with further loss of compensatory hyperinsulinemia. Rap treatment partially decreased plasma triglyceride levels (Fig. 4C) and this is consistent with previous reports. Heart weight (adjusted to tibia length) was further increased in 15-week ZDF rats (Fig. 4D) compared to the heart weight of 11-week ZDF rats (Fig. 3H). Rap treatment reduced heart weight partially in 15-week ZDF rats (Fig. 4D). Suppression of hypertrophy by Rap has been previously reported [36] and the observed reduction of heart weight in 15-week ZDF rat is consistent with this effect.

However, qRT-PCR showed that Rap treated ZDF rats had at least a 2-fold increase in the expression of all miR-29 family members (miR-29a, b and c) (Fig. 4E). qRT-PCR analysis of MCL-1 mRNA levels in Rap-treated ZDF rats showed that there was at least a 4-fold suppression of cardiac MCL-1 mRNA expression in response to Rap treatment (Fig. 4F). These observations suggest that Rap treatment causes severe dysregulation of the miR-29-MCL-1 axis in cardiac tissues of ZDF rat.
show loss of MCL-1 did not promote apoptosis (16, 17). This observation is consistent with previous reports that detectable apoptosis was found in the heart tissues from either control or Rap-treated ZDF rats whereas positive controls for paraffin embedded sections of right ventricle by using ApopTag Fluorescein In Situ Apoptosis Detection Kit S7110 (Millipore). No observable differences in the right ventricle tissues of Rap treated ZDF rats and control ZDF rats. These observations suggest that the myocardium of Rap-treated ZDF rats that had a further increase in miR-29 a, b and c miRNAs and further suppression of MCL-1 (Fig. 4E and 4F) compared to age-matched control rats, exhibited significant disorganization of myofibril bundles that reflect tissue damage. That was not observed in age-matched control rats. To identify if apoptosis is associated with the disorganization of cardiomyocytes, we performed an indirect TUNEL assay on the formalin fixed, paraffin embedded sections of right ventricle by using ApoTag Fluorescein In Situ Apoptosis Detection Kit S7110 (Millipore). No detectable apoptosis was found in the heart tissues from either control or Rap-treated ZDF rats whereas positive controls for apoptosis exhibited detectable apoptosis by this method (data not shown). This observation is consistent with previous reports that show loss of MCL-1 did not promote apoptosis (16, 17).

Discussion

Despite extensive epidemiological evidence that suggests DM is an independent predictor of heart disease and heart failure, the exact mechanisms by which DM causes cardiac damage is not clear. An increasing detection of asymptomatic left ventricular dysfunction without overt cardiac disease is noted in diabetic patients as well [37]. However, increased rate of sudden cardiac death is associated with DM and the mechanisms underlying this pathology are unclear [38].

A recent study has reported that in 14-week old ZDF rats there is a significant decrease in RV and LV function compared to age and gender matched ZL rats. Moreover, a similar decrease in RV and LV metabolic rates of glucose utilization measured under hyperinsulinaemic euglycaemic conditions was found in ZDF rats compared to ZL rats [5]. Interestingly, it was also observed that RV myocardium of 12–17 week old ZDF rats did not show any significant structural damage despite the fact that myocardial impulse propagation was impaired in the RV tissues [6]. However, myocardium of about 32 week (8-month) old ZDF rats showed a loss of myocytes [39]. Thus, an intriguing question arises regarding what the mechanisms that contribute to cardiomyocyte loss in DM are, and whether they are just an effect of aging in the background of DM. Many studies have shown that Rap treatment promotes the development of DM [18–24]. Therefore, our rationale was that treating ZDF rats with Rap would expedite their DM progression and this would give us an opportunity to compare ZDF rats in the same age group, but exhibiting pathology associated with advanced stages of DM. Such an approach would help to subtract the age factor from the group of factors that contribute to the cardiomyocyte disorganization and loss associated with progression of DM.

Increased expression of diabetic marker miR-29 family miRNAs is seen in rodent models of DM and in young and adult diabetic patients with T1DM or T2DM. We undertook this study to uncover the role of microRNA miR-29 family and its target MCL-1, a pro-survival molecule that is critical for cardiomyocyte survival under stress, in the myocardium damage seen in diabetic heart disease. For this study, we focused on the RV of ZDF rat heart since RV dysfunction from structural and functional perspectives has been described previously in young ZDF rats [5,6] and therefore the baseline parameters were easy to compare in the context of regulation of the miR-29-MCL-1 axis. Moreover, evaluation of RV myocardium damage serves as a strong indicator of advanced heart disease in young ZDF rats since RV heart failure typically follows LV heart failure.

Our in vitro studies on mouse cardiomyocyte HL-1 cells showed that insulin regulates miR-29 family miRNAs (mir-29a, b and c) and improves cardioprotective MCL-1 levels in cardiomyocytes.
Conversely, inhibition of mTORC1 signaling resulted in up-regulation of miR-29 expression and suppressed MCL-1 expression in cardiomyocytes. These observations revealed that a miR-29-MCL-1 axis exists in cardiomyocytes. Therefore, we investigated if dysregulation of miR-29-MCL-1 axis is correlated to cardiac damage in DM.

First, we tested the status of cardiac miR-29-MCL-1 axis in the RV tissues of ZDF rats at the age of 11-weeks compared to age-matched ZL rats. Consistent with previous reports [34,35], ZDF rats showed hyperinsulinemia, hyperglycemia, hyperlipidemia, and increased body weight compared to age-matched lean rats. They also had increased heart weight and reduced lean muscle
Conclusions

Data presented here shows that insulin down-regulates the expression of diabetic marker miR-29 family miRNAs in mouse cardiomyocytes and preserves the expression of cardioprotective MCL-1. Consistent with this insulin effect, 11-week old hyperinsulinemic ZDF rats only had a mild loss of MCL-1 expression and did not show any damage in myocardium. Therefore, we conclude that regulation of miR-29-MCL-1 axis by insulin is a cardioprotective mechanism and compensatory hyperinsulinemia in conditions of hyperglycemia would regulate miR-29-MCL-1 axis in diabetics. Moreover, hyperinsulinemia could protect against myocardial damage in DM patients.

Collectively, our findings suggest that insulin down-regulates the expression of diabetic marker miR-29 family miRNAs in mouse cardiomyocytes and preserves the expression of cardioprotective MCL-1. This suggests that insulin may be a potential therapeutic target for the treatment of diabetic heart disease.
vulnerability of diabetic heart to sudden malfunction that results in death.

Rap is known to inhibit pancreatic β-cell proliferation and β-cell adaptation to hyperglycemia. Consistent with this effect of Rap, we observed a significant suppression of fasting plasma insulin levels in Rap-treated ZDF rats. Future studies will investigate how Rap-treatment modulates pancreatic weight, pancreatic insulin content, and β-cell mass and function in ZDF rats.

In this study we used mouse atrial cardiomyocyte HL-1 cells and right ventricular tissues of ZDF rats to investigate how Rap modulates expression of miR-29-MCL-1 axis. Further studies with primary cultures of cardiomyocytes from rat atrium and ventricle are needed to confirm that INS-mediated modulation of miR-29-MCL-1 axis is similar in atrial and ventricular cells. We have shown in vitro that a miR-29 inhibitor cocktail could reverse Rap-mediated suppression of MCL-1 protein expression in cardiomyocytes. Further studies are needed to also confirm that in vivo rodent models a miR-29 inhibitor cocktail would improve cardiac MCL-1 protein expression.

Based on the data presented here, we contend that the normal functioning of miR-29-MCL-1 axis is an important cardioprotective mechanism regulated by insulin that exists in female mouse atrial cardiomyocytes and male ZDF rat heart tissue. The extent of loss of this mechanism in response to the progression of DM may determine the extent of cardiac damage as seen in our control versus Rap-treated ZDF rat models (Fig. 6). This observation may have important clinical relevance given the fact that patients with DM are reported to have an increase in miR-29 expression [5,44]. DM patients are often treated with mTORC1 inhibitors as part of prophylaxis for organ transplant procedures. Though rapamycin has well-established cardioprotective effects, an additional increase in miR-29 family miRNAs due to mTORC1 inhibition in the heart tissues of DM patients can potentially suppress MCL-1 and exacerbate cardiomyocyte disorganization and cardiac damage.

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Author Contributions

Conceived and designed the experiments: NA LP. Performed the experiments: LP NA CL RG PK. Analyzed the data: LP NA CL RG PK. Contributed reagents/materials/analysis tools: LP. Wrote the paper: LP NA CL RG PK.

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