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MicroRNA-195 promotes palmitate-induced apoptosis in cardiomyocytes by down-regulating Sirt1

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Aims Free fatty acids induce apoptosis in cardiomyocytes, which is implicated in lipotoxic cardiomyopathy. However, the underlying mechanisms remain not fully understood. MicroRNAs (miRNAs) are non-coding small RNAs that control gene expression at the post-transcriptional level. Dysregulated miRNAs have been shown to be involved in heart diseases. This study was to examine whether miR-195 regulates palmitate-induced cardiomyocyte apoptosis by targeting Sirt1, a known anti-apoptotic protein.

Methods and results In cultured neonatal mouse cardiomyocytes, palmitate up-regulated miR-195 expression, increased reactive oxygen species (ROS) production, and induced apoptosis as determined by up-regulation of caspase-3 activity and DNA fragmentation. Inhibition of miR-195 decreased ROS production and apoptosis in palmitate-stimulated cardiomyocytes. In contrast, a miR-195 mimic enhanced palmitate-induced ROS production and apoptosis. The induction of miR-195 correlated with a reduction in Sirt1 and Bcl-2. We further showed that miR-195 targeted and inhibited Sirt1 expression through two target sites located in the 3′ un-translational region of Sirt1 mRNA. In concordance, inhibition of miR-195 increased Sirt1 protein in cardiomyocytes whereas the miR-195 mimic reduced it. Activation of Sirt1 or overexpression of Bcl-2 inhibited palmitate-induced apoptosis. On the other hand, inhibition of Sirt1 enhanced apoptosis. The inhibitory effect of Sirt1 on apoptosis was associated with a reduction in ROS.

Conclusions This study demonstrates a pro-apoptotic role of miR-195 in cardiomyocytes and identifies Sirt1 as a direct target of miR-195. The effect of miR-195 on apoptosis is mediated through down-regulation of Sirt1 and Bcl-2 and ROS production. Thus, miR-195 may be a new therapeutic target for lipotoxic cardiomyopathy.

Keywords Apoptosis • Cardiomyocytes • miRNA-195 • Sirt1 • Palmitate

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1. Introduction

Saturated free fatty acids such as palmitate induce apoptosis in cardiomyocytes, which has been implicated in cardiac dysfunction in obesity and diabetes.[1–4] The induction of apoptosis by palmitate has been associated with the mitochondria-dependent apoptotic pathway including cytochrome *c* release and loss of the mitochondrial membrane potential and consequent caspase-3 activation in cardiomyocytes.[5,6] However, the exact mechanisms remain not fully understood.

MicroRNAs (miRNAs) are a class of short RNA molecules, on average 22 nucleotides long, encoded within the genome and derived from endogenous small hairpin precursors.[7,8] The miRNAs negatively regulate gene expression by targeting the 3′ un-translational region (3′UTR) of specific messenger RNA (mRNA) for transcript degradation or translational repression.[9,10] miRNAs are involved in

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a wide range of pathophysiological cellular processes including development, differentiation, growth, metabolism, survival/death, and tumour formation.[11–15] Aberrant expression of miRNAs has been linked to a number of myocardial pathological conditions including hypertrophy, fibrosis, apoptosis, regeneration, arrhythmia, and heart failure.[16–20] As such, miRNAs have been suggested as novel therapeutic targets for heart diseases. Previous studies have demonstrated that miR-195 is associated with cardiac hypertrophy and heart failure. Forced overexpression of miR-195 is sufficient to induce cardiac hypertrophy and heart failure in transgenic mice.[21] However, it remains to be determined whether miR-195 plays a role in cardiomyocyte apoptosis.

Sirtuin 1 (Sirt1), known as NAD-dependent deacetylase, belongs to class III histone/protein deacetylases and is a member of the silent information regulator (Sir2) family. Sirt1 plays a pivotal role in a wide variety of cellular processes such as apoptosis/cell survival, endocrine signalling, chromatin remodelling, and gene transcription.[22,23] Recent studies have suggested that Sirt1 is an important endogenous apoptosis inhibitor in cardiomyocytes.[24] However, it is unclear whether Sirt1 also protects cardiomyocytes against apoptosis induced by palmitate and whether miR-195 targets Sirt1 in cardiomyocytes.

The purpose of this study was to investigate the role of miR-195 and Sirt1 in cardiomyocytes apoptosis in response to palmitate, and to examine whether miR-195 regulates Sirt1 expression in palmitate-stimulated cardiomyocytes.

2. Methods

2.1 Animals

This investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23). All experimental procedures were approved by the Animal Use Subcommittee at the University of Western Ontario, Canada. Breeding pairs of C57BL/6 mice were purchased from the Jackson Laboratory to produce neonates for cardiomyocyte isolation.

2.2 Neonatal mouse cardiomyocyte culture

Neonatal mice (Born within 2 days) were euthanized by decapitation and the neonatal cardiomyocytes were prepared and cultured according to methods we described previously.[25]

2.3 Drugs

Palmitate, oleate, *N*-acetylcysteine (NAC), nicotinamide, and resveratrol were purchased from Sigma or Calbiochem. The mitochondria-targeted antioxidant peptide SS31 (H-D-Arg-Dmt-Lys-Phe-NH_2, Dmt = 2′,6′-dimethyltyrosine) and peptide SS20 (H-Phe-D-Arg-Phe-Lys-NH_2), which lacks antioxidant properties were synthesized using a published protocol, as previously described.[26]

2.4 Gene knockdown using small interfering siRNA

In order to knockdown Sirt1 expression, a small interfering RNA (siRNA) against mouse Sirt1 was obtained (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a scramble siRNA was employed as a control. Transfection was performed using TransMessenger Transfection Reagent (Qiagen) according to the manufacturer's instructions as described previously.[27,28]

2.5 Modulation of miR-195

A chemically modified antisense oligonucleotide (antagomir, GenePharm Co. Ltd,) and a synthetic miR-195 mimic (Qiagen) were used to inhibit and overexpress miR-195 expression, respectively. A scrambled oligonucleotide (GenePharm Co. Ltd,) was used as a control. Transfection was performed by using TransMessenger transfection reagent (Qiagen) according to the manufacturer's instructions as described previously.[27,28]

2.6 miR-195 expression assay

Total RNA was extracted from neonatal mouse cardiomyocytes using TRIzol reagent (Invitrogen). miR-195 expression was determined by using the miRNA plate assay kit (Signosis, Inc.,) according to the manufacturer's instructions. U6 was used as an internal control.

2.7 Active caspase-3

As described in detail previously,[28] caspase-3 activity in cardiomyocytes was measured by using a caspase-3 fluorescent assay kit (BIOMOL Research Laboratories).

2.8 Western blot analysis

The protein levels of Sirt1, Bcl-2, cleaved caspase-3, and GAPDH were determined by western blot analysis using respective specific antibodies (Cell Signaling).

2.9 Intracellular reactive oxygen species measurement

The production of reactive oxygen species (ROS) was measured by using the ROS sensitive dye, 2,7-dichlorodihydro-fluorescein diacetate (DCF-DA, Invitrogen), as an indicator. The assay was performed as we described in our recent report.[29] Briefly, cardiomyocytes were homogenized in assay buffer. The homogenates were incubated with DCF-DA at 37°C for 3 h. The fluorescent product formed was quantified by spectrofluorometer at the 485/525 nm. Changes in fluorescence were expressed in arbitrary units.

2.10 Measurement of cellular DNA fragmentation

Cardiomyocytes were pre-labelled with BrdU and then incubated with palmitate. DNA fragmentation was measured using a Cellular DNA Fragmentation ELISA kit (Roche Applied Science) according to the manufacturer's instructions.

2.11 Plasmids

The luciferase vector including 3′ UTR of human Sirt1 containing the Sirt1-miR-195 response elements (wt-Luc-Sirt1) was purchased from Addgene Inc.[30] A mutant within the two Sirt1-miR-195 response elements of 3′ UTR of Sirt1 (mu-Luc-Sirt1) was generated by using site-directed gene mutagenesis, whose sequences contained 5′-UAAUAUUUU GGACugcugUU-3′ (the five lowercase nucleotides are deleted) and 5′-TAAAGTATTCCTCTGT***ACGA***T-3′ (the four bold and italic nucleotides are substitutes for TGCT). The reporter vector consisting of a luciferase gene followed by the miR-195 binding consensus sequence was purchased from Signosis, Inc. (Sunnyvale, CA, USA). Plasmid expressing human Bcl-2 (*p*CMV-Bcl2) was purchased from Addgene Inc.[31]

2.12 Luciferase assays

Cardiomyocytes were cultured for 24 h. Two hundred nanograms of plasmid DNA (wt-Luc-Sirt1 or mu-Luc-Sirt1) and miR-195 mimic, miR-195 antagomir, or a scrambled oligonucleotide were co-transfected by using Attractene Transfection Reagent (Qiagen) according to the manufacturer's instructions. The *p*RL-CMV vector containing the CMV enhancer and early promoter elements to provide high-level expression of

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Renilla luciferase (Promega) served as an internal control. Luciferase assays were performed by using the dual luciferase reporter assay system (Promega) 24 h after transfection.

2.13 Statistical analysis

All data were given as MEAN ± SD. ANOVA followed by Newman–Keuls test was performed for multigroup comparisons. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1 Palmitate up-regulates miR-195 expression in cardiomyocytes

Purity of cardiomyocytes after 72 h of cell culture was determined by immunocytochemical staining using an antibody specific for cardiac troponin-I. Purity of cardiomyocytes was >90%, which is consistent with previous reports.[32] Thus, the contamination of other cell types including fibroblasts and endothelial cells was very limited.

To investigate the miR-195 expression in cardiomyocytes in response to saturated fatty acids, neonatal mouse cardiomyocytes were incubated with palmitate or oleate (0.1 mM) as a control. Twenty-four hours later, miR-195 was determined in cardiomyocytes using the miRNA plate assay. As shown in *Figure 1A*, miR-195 was significantly increased in palmitate- compared with oleate-stimulated cardiomyocytes. However, oleate treatment did not alter miR-195 expression in cardiomyocytes (data not shown). To further confirm the up-regulation of miR-195 by palmitate, we co-transfected cardiomyocytes with a reporter vector consisting of a luciferase gene followed by the miR-195 binding consensus sequence and *p*RL-CMV as an internal control, and then exposed them to palmitate or oleate. Inclusion of the miR-195 binding consensus sequence within the 3′UTR of a luciferase gene renders it a target of miR-195. Twenty four hours after palmitate treatment, the luciferase activity was measured in cardiomyocytes. Exposure to palmitate significantly reduced the luciferase activity (*Figure 1B*), suggesting an increase in miR-195 expression as miR-195 represses luciferase expression. These results demonstrate that palmitate induces miR-195 expression in cardiomyocytes.

3.2 Induction of miR-195 promotes apoptosis in palmitate-stimulated cardiomyocytes

To determine the role of miR-195 in apoptosis, cardiomyocytes were transfected with miR-195 antagomir or a scrambled oligonucleotide as a control. Twenty-four hours later, cardiomyocytes were incubated with palmitate, oleate (0.1 mM) or vehicle for another 24 h. Apoptosis was then determined by caspase-3 activity, cleaved caspase-3, and DNA fragmentation. Oleate treatment did not have any significant effect on apoptosis in cardiomyocytes (data not shown) and thus, in the following studies, we did not include un-treated control group. Consistent with previous studies,[5,6] palmitate treatment induced caspase-3 activation and DNA fragmentation in cardiomyocytes compared with oleate (*Figure 1C–G*). However, these effects of palmitate on apoptosis were significantly attenuated by miR-195 antagomir (*Figure 1C–E*). To further demonstrate the role of miR-195, we introduced miR-195 mimic into cardiomyocytes and then incubated these cells in palmitate or oleate for 24 h. Similarly, the miR-195 mimic further enhanced caspase-3 activity and DNA fragmentation (*Figure 1F–G*). Both miR-195 mimic and antagomir did not have any effect on basal apoptosis in cardiomyocytes (data not shown). Since most contaminated non-cardiomyocytes in neonatal cardiomyocyte culture are fibroblast cells, we incubated cardiac fibroblast cells with palmitate or oleate (0.1 mM). Twenty-four hours later, palmitate incubation did not induce apoptosis (data not shown). Thus, these results indicate that miR-195 promotes apoptosis in palmitate-stimulated cardiomyocytes.

3.3 miR-195 induces reactive oxygen species production which contributes to apoptosis

Palmitate has been shown to induce ROS production in cardiomyocytes. In agreement with this previous report,[33] ROS production was also increased in palmitate- compared with oleate-stimulated cardiomyocytes (*Figure 2A–B*). To examine whether miR-195 contributes to ROS production, we transfected cardiomyocytes with miR-195 antagomir, mimic or a scrambled oligonucleotide. Twenty-four hours after transfection, cardiomyocytes were incubated with palmitate or oleate for another 24 h and ROS production was measured in cardiomyocytes. miR-195 antagomir reduced, whereas miR-195 mimic enhanced ROS production in palmitate stimulated cardiomyocytes (*Figure 2A–B*). However, both miR-195 mimic and antagomir did not have any effect on basal ROS production in cardiomyocytes. This result demonstrates that miR-195 promotes ROS production.

To determine whether ROS production is involved in palmitate-induced apoptosis, we incubated cardiomyocytes with palmitate or oleate in the presence of NAC (2.5 or 5 mM), an antioxidant and glutathione precursor reported to effectively reduce ROS generation.[34] Twenty-four hours later, caspase-3 activity and DNA fragmentation were measured in cardiomyocytes. NAC dose-dependently reduced caspase-3 activity and DNA fragmentation during palmitate stimulation (*Figure 2C–D*). To further demonstrate the effect of ROS production on apoptosis, we used the mitochondrial targeted antioxidant peptide SS31. SS31 specifically quenches mitochondrial ROS.[35] The structurally related peptide SS20, which lacks antioxidant properties, served as a control. Cardiomyocytes were incubated with palmitate in the presence of SS31 or SS20 (2.5 μM) for 24 h. Incubation with SS31 significantly reduced caspase-3 activity and DNA fragmentation in palmitate-stimulated cardiomyocytes (*Figure 2E–F*). This result suggests that selectively blocking mitochondrial ROS prevents palmitate-induced apoptosis. Taken together, these findings indicate that ROS production, at least in part from mitochondria, contributes to apoptosis in palmitate-stimulated cardiomyocytes.

3.4 miR-195 targets and inhibits Sirt1

Palmitate has been shown to reduce Sirt1 protein expression in monocytes.[36] In agreement with this previous report, the levels of Sirt1 protein were also decreased in palmitate-stimulated cardiomyocytes (*Figure 3A*), which correlated with an increase in miR-195 expression. This result suggests that miR-195 may target and down-regulate Sirt1 protein expression. Indeed, transfection of the miR-195 mimic significantly reduced Sirt1 protein levels in cardiomyocytes (*Figure 3B*). On the other hand, miR-195 antagomir increased Sirt1 protein in cardiomyocytes (*Figure 3C*). These results suggest that miR-195 negatively regulates Sirt1 protein expression in cardiomyocytes.

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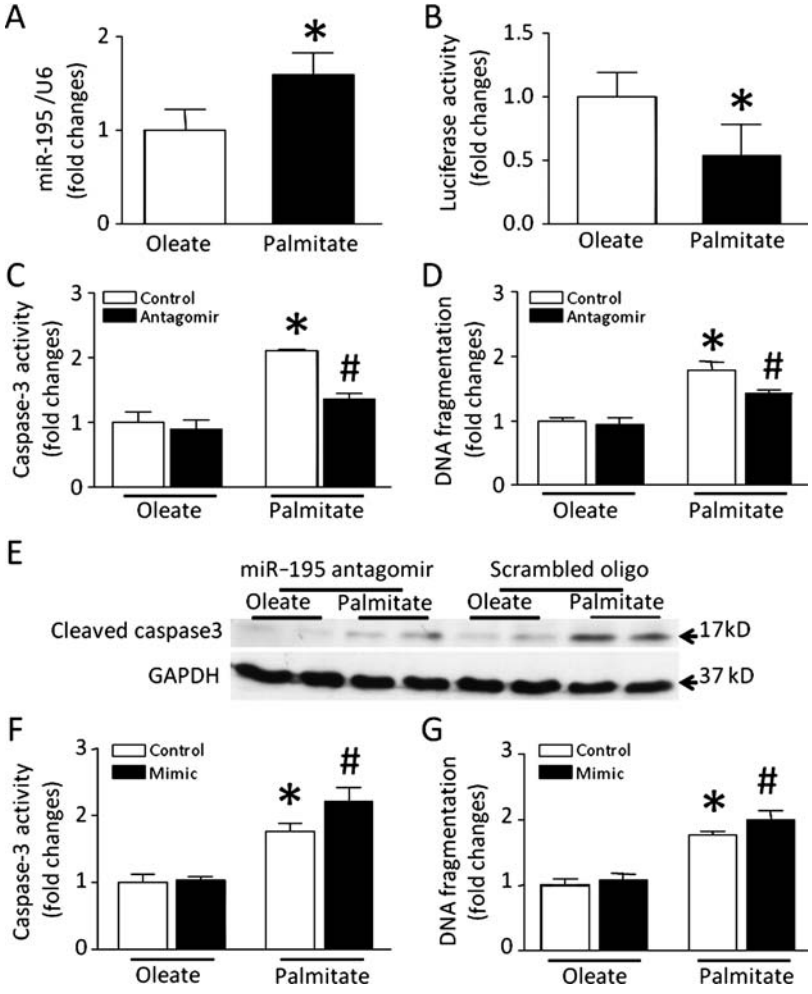

Figure 1 miR-195 expression and its role in apoptosis. (*A*) Cultured neonatal cardiomyocytes were incubated with palmitate or oleate (0.1 mM) for 24 h and miR-195 was determined by using the miRNA plate assay kit. Palmitate increased miR-195 in cardiomyocytes. (*B*) Cardiomyocytes were co-transfected with a reporter vector consisting of a luciferase gene followed by the miR-195 binding consensus sequence and pRL-CMV as an internal control, and then exposed to palmitate or oleate. The luciferase activity was measured in cardiomyocytes. Palmitate induced miR-195 since inclusion of miR-195 binding consensus sequence within the 3′UTR of a luciferase gene renders it a target of miR-195. Data are mean ± SD from three different cell cultures. $*P < 0.05$ vs. oleate. (*C–G*) Cardiomyocytes were transfected with miR-195 antagomir, mimic or a scrambled oligonucleotide as a control, and then incubated with palmitate or oleate for 24 h. Caspase-3 activity, cleaved caspase-3 and cellular DNA fragmentation were determined in cardiomyocytes. (*C–D*) miR-195 antagomir inhibited caspase-3 and reduced DNA fragmentation in palmitate-stimulated cardiomyocytes ($n = 3$ in each group). (*E*) A representative western blot for the cleaved caspase-3 protein (17/19 KD) from three different cell cultures in each group (duplicate for each cell culture) shows palmitate increased the levels of cleaved caspase-3 protein and miR-195 antagomir decreased cleaved caspase-3 protein during palmitate incubation. (*F–G*) miR-195 mimic enhanced caspase-3 activity and DNA fragmentation in palmitate-stimulated cardiomyocytes ($n = 5$ in each group). Data are mean ± SD. $*P < 0.05$ vs. oleate in control; $\#P < 0.05$ vs. Palmitate in Control.

It is generally accepted that miRNAs negatively regulate gene expression by targeting the 3′ UTR of specific mRNAs and inducing their degradation and/or translational repression. For this reason, we identified two putative binding sites located in the 3′UTR of Sirt1 by using the TargetScan5 software. As shown in *Figure 3D*, the alignments between miR-195 and two regions within the 3′UTR of human Sirt1 represent two different putative target sequences that can confer inhibition of translation by miR-195. To clarify whether Sirt1 is a direct target of miR-195, we used a reporter vector containing a luciferase gene followed by the 3′ UTR of human Sirt1 mRNA (wt-Luc-Sirt1). Overexpression of miR-195 by its mimic inhibited the luciferase activity in wt-Luc-Sirt1 transfected cardiomyocytes (*Figure 3E*). To verify this, we mutated these two miR-195 putative-binding sites on the 3′UTR of Sirt1 of wt-Luc-Sirt1 by either deletion or substitution (*Figure 3D2*). The mutation abrogated the inhibitory effect of miR-195 on the luciferase activity in cardiomyocytes (*Figure 3E*). For further confirmation, we transfected cardiomyocytes with wt-Luc-Sirt1 or mutated one (mu-Luc-Sirt1) and exposed these cells with palmitate or oleate. Palmitate significantly inhibited the luciferase activity in wt-Luc-Sirt1 but not in mu-Luc-Sirt1 transfected cardiomyocytes (*Figure 3F*). These results strongly support the view that miR-195 directly targets and inhibits Sirt1 expression

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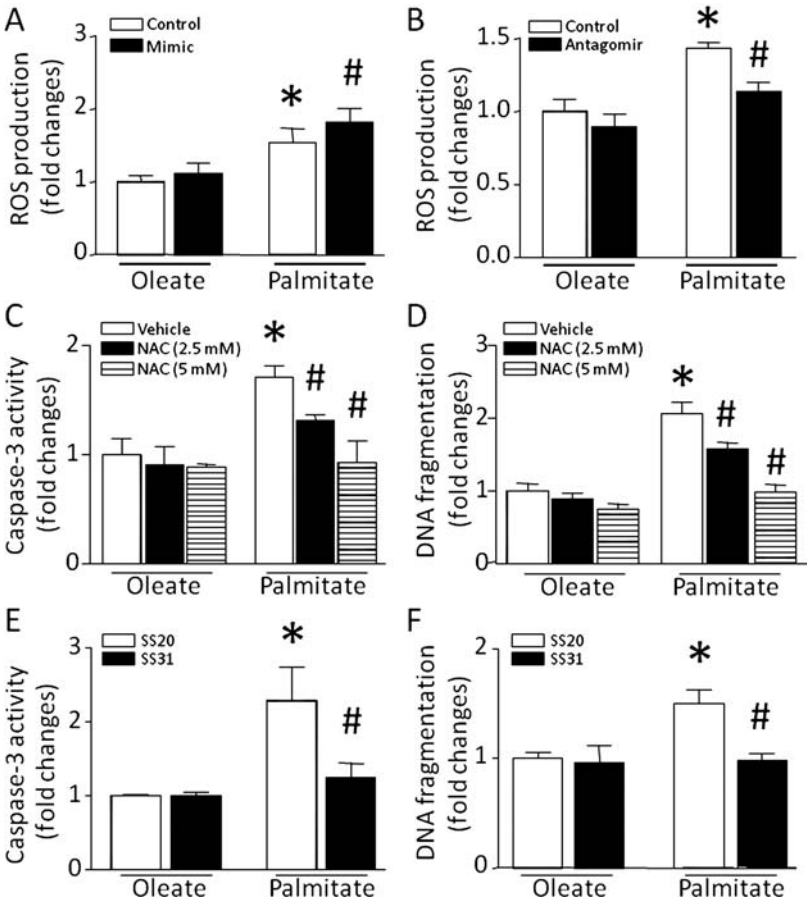

Figure 2 Role of miR-195 in ROS production and effect of ROS inhibition on apoptosis. (*A–B*) Cardiomyocytes were transfected with miR-195 antagomir, mimic, or a scrambled oligonucleotide as a control, and then incubated with palmitate or oleate for 24 h. Intracellular ROS production was measured ($n = 5$ in each group). (*C–D*) Effect of NAC on apoptosis. Cardiomyocytes were incubated with palmitate or oleate in the presence of NAC (2.5 or 5 mM) or vehicle for 24 h. Caspase-3 activity (*C*) and DNA fragmentation (*D*) were determined in cardiomyocytes ($n = 3$ in each group). (*E–F*) Effect of SS31 on apoptosis. Cardiomyocytes were incubated with palmitate or oleate in the presence of SS31 or SS20 (2.5 μM) as a control for 24 h and caspase-3 activity (*E*) and DNA fragmentation (*F*) were then measured in cardiomyocytes ($n = 3$ in each group). Data are mean ± SD. *$P < 0.05$ vs. control, vehicle or SS20 in oleate; #$P < 0.05$ vs. control, vehicle or SS20 in Palmiate.

in cardiomyocytes. Since two putative sites for miR-195 were identified in the 3′ UTR of human Sirt1, whereas our experiments were conducted in mouse cardiomyocytes, we compared the degree of conservation of these sites in human and mouse Sirt1, and found that the first site, but not the second is highly conserved between human and mouse. To target the two sites independently, we mutated the first or second miR-195 putative-binding sites alone as described above. After co-transfection, either of the mutations abrogated the inhibitory effect of miR-195 antagomir on the luciferase activity (data not shown). This result suggests that either site alone is sufficient in the regulation of Sirt1 expression.

3.5 Sirt1 prevents reactive oxygen species production and inhibits apoptosis

Having shown that miR-195 promotes apoptosis and inhibits Sirt1 expression, we then investigated the role of Sirt1 in apoptosis in palmitate-stimulated cardiomyocytes. In this regard, we first incubated cardiomyocytes with palmitate in the presence of the well-known Sirt1 activator, resveratrol (5 μM) or vehicle. Apoptosis inhibition by resveratrol was indicated by the reduction in caspase-3 activity and DNA fragmentation determined at the end of the 24-h incubation period (*Figure 4A–B*). Similarly, palmitate-induced ROS production was also decreased by resveratrol in cardiomyocytes (*Figure 4C*). To further demonstrate the role of Sirt1, we knocked down Sirt1 using siRNA. Cardiomyocytes were transfected with Sirt1 siRNA and then incubated with palmitate or oleate. A scrambled siRNA was used as a control. Transfection with siRNA significantly reduced Sirt1 protein (*Figure 4D*), confirming a successful knockdown of Sirt1. Down-regulation of Sirt1 had no effect on basal apoptosis but significantly enhanced palmitate-induced apoptosis, as determined by increases in caspase-3 activity and DNA fragmentation (*Figure 4E–F*). Knockdown of Sirt1 also increased palmitate-stimulated but not basal ROS production in cardiomyocytes (*Figure 4G*). These effects of Sirt1 inhibition on apoptosis and ROS production were also examined by using a pharmacological Sirt1 inhibitor, nicotinamide. Consistently, incubation in the presence of nicotinamide (50 μM) exacerbated apoptosis and ROS production in response to

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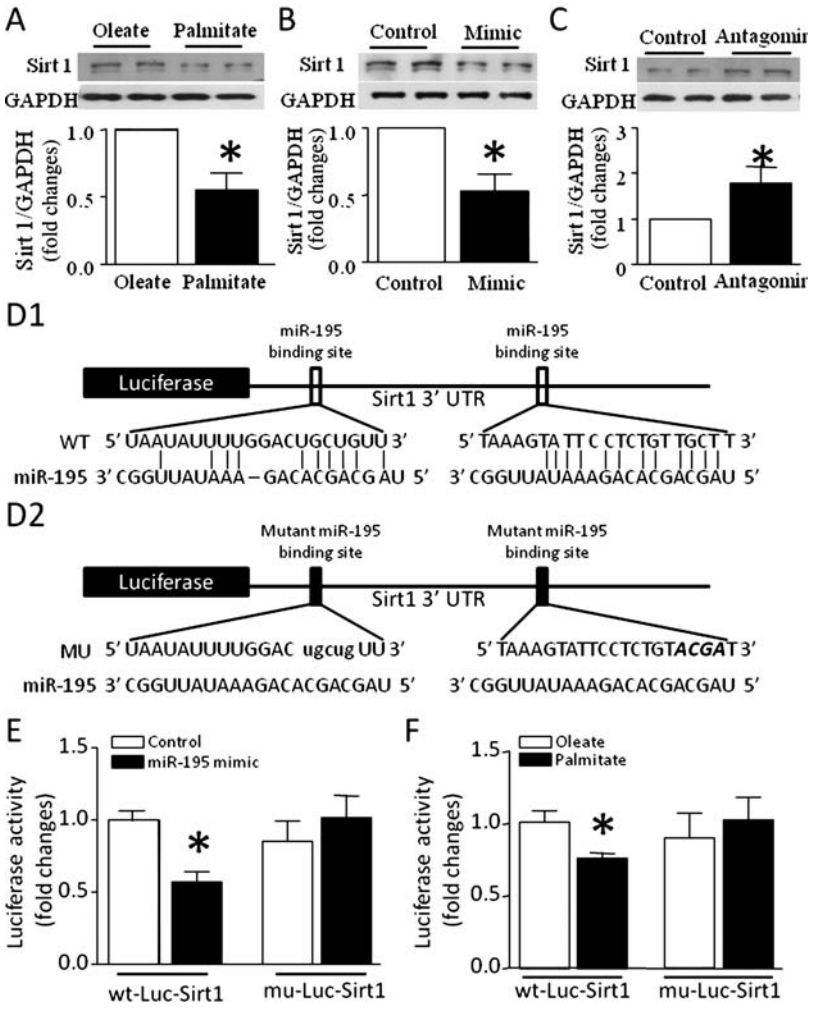

Figure 3 Effect of palmitate and role of miR-195 in Sirt1 expression. Cardiomyocytes were transfected with miR-195 antagomir, mimic, or a scrambled oligonucleotide as a control, or incubated with palmitate or oleate. Twenty-four hours later, Sirt1 protein was determined by western blot analysis. (*A*) Palmitate decreased Sirt1 protein. (*B*) miR-195 mimic reduced Sirt1 protein. (*C*) miR-195 antagomir increased Sirt1 protein. Upper panel is the representative western blot for Sirt1 protein and lower panel is the quantification of Sirt1 protein. (*D*) Diagram of plasmid construction. (*D1*) A segment of Sirt1 3′UTR was inserted downstream of the luciferase-coding sequence. Sequence alignment of miR-195 and 3′UTR of Sirt1 shows the complementarity at the 5′ end of miR-195, where the crucial seed region is located. (*D2*) Sequence alignment of miR-195 and mutated 3′UTR of Sirt1 shows no complementarity at the 5′ end of miR-195. The five lowercase nucleotides are deleted and the four bold and italic nucleotides are substitutes for TGCT. (*E*) Cardiomyocytes were co-transfected with the plasmid containing the segment of wild-type 3′UTR of Sirt1 (wt-Luc-Sirt1) or containing the segment of mutated 3′UTR of Sirt1 (mu-Luc-Sirt1), and miR-195 mimic or a scrambled oligonucleotide as a control. Dual luciferase activity assay was performed in cardiomyocytes. (*F*) Cardiomyocytes were transfected with the plasmid wt-Luc-Sirt1 or mu-Luc-Sirt1, and then incubated with palmitate or oleate for 24 h. Dual luciferase activity assay was performed in cardiomyocytes. Data are MEAN ± SD from three different cell cultures. *$P < 0.05$ vs. control or oleate.

palmitate (*Figure 5A–C*). Thus, Sirt1 prevents ROS production and inhibits apoptosis in palmitate-stimulated cardiomyocytes.

To further demonstrate that down-regulation of Sirt1 is one of the mechanisms by which miR-195 induces apoptosis, we transfected cardiomyocytes with miR-195 antagomir or a scrambled oligonucleotide as a control and then incubated them with palmitate in the presence of nicotinamide or vehicle for 24 h. Consistently, miR-195 antagomir reduced apoptosis and ROS production. However, these effects of miR-195 antagomir were significantly attenuated by nicotinamide (*Figure 5D–F*). This result further supports the view that the role of miR-195 in apoptosis is mediated, at least partly, through down-regulation of Sirt1.

3.6 miR-195 targets Bcl-2 in palmitate-induced apoptosis

Bcl-2 has been recently shown to be a target of miR-195.[37] We therefore determined Bcl-2 protein expression in palmitate-treated cardiomyocytes. As shown in *Figure 6A*, palmitate induced a reduction in Bcl-2 protein, which was restored by miR-195 antagomir. This suggests that miR-195 may also target and repress Bcl-2 expression in cardiomyocytes. To examine the role of Bcl-2 in palmitate-induced apoptosis, we transfected rat H9C2 cardiac muscle cells with a plasmid expressing human Bcl-2 (*p*CMV-Bcl2, Addgene, Inc.,) or an empty plasmid as a control. After transfection, the cells were

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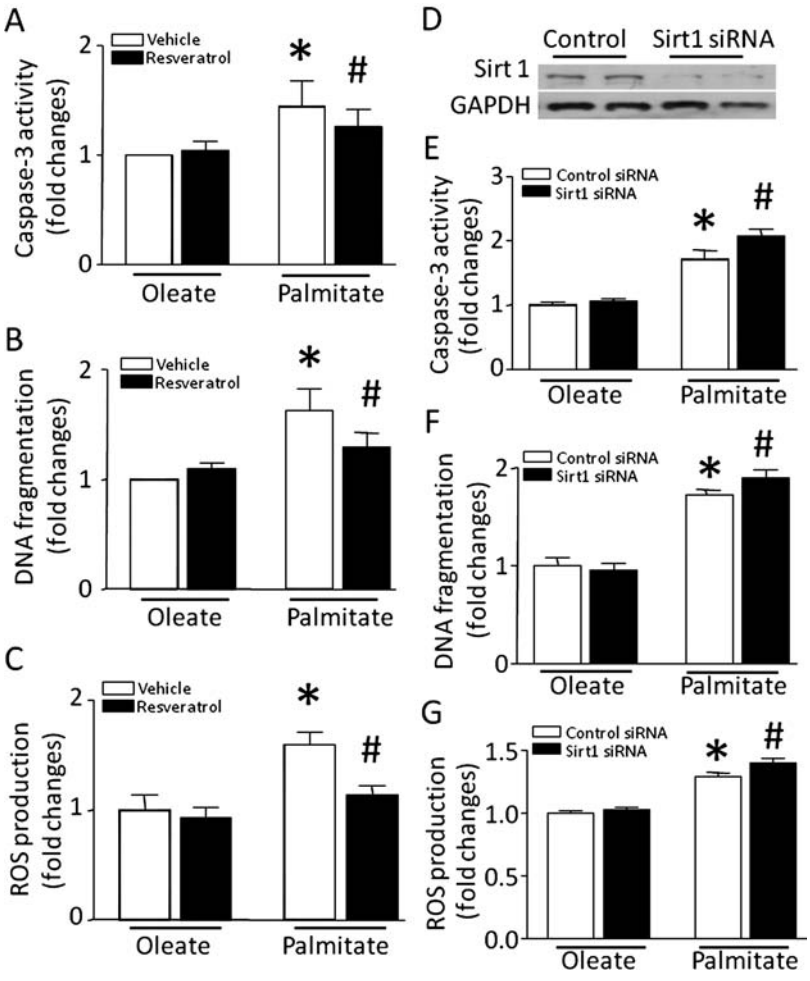

Figure 4 Effects of resveratrol and Sirt1 siRNA on apoptosis and ROS production. Cardiomyocytes were incubated with palmitate or oleate (0.1 mM) in the presence of resveratrol (5 μM) or vehicle for 24 h. Caspase-3 activity (*A*), DNA fragmentation (*B*), and intracellular ROS production (*C*) were determined in cardiomyocytes. Data are MEAN ± SD from three different cell cultures. *$P < 0.05$ vs. oleate in vehicle; #$P < 0.05$ vs. palmitate in vehicle. (*D*–*G*) Cardiomyocytes were transfected with Sirt1 siRNA or a scrambled siRNA as a control and then incubated with palmitate or oleate for 24 h. (*D*) A representative western blot from three different cell cultures (duplicate in each group) shows down-regulation of Sirt1 protein by siRNA in cardiomyocytes. Caspase-3 (*E*), DNA fragmentation (*F*), and intracellular ROS (*G*) were measured in cardiomyocytes. Data are MEAN ± SD from four different cell cultures. *$P < 0.05$ vs. oleate in control siRNA or vehicle; #$P < 0.05$ vs. Palmitate in control siRNA or vehicle.

incubated with palmitate or oleate (0.1 mM). Caspase-3 activity was significantly increased in palmitate-stimulated H9C2 cells. However, transfection with pCMV-Bcl2 reduced caspase-3 activity (*Figure 6B*). This result demonstrates that Bcl-2 prevents apoptosis in palmitate-induced cardiomyocytes.

4. Discussion

The major findings of this study are that induction of miR-195 expression promotes apoptosis and ROS production in palmitate-stimulated cardiomyocytes. miR-195 directly targets Sirt1 and represses Sirt1 expression. Sirt1 inhibits ROS production and protects cardiomyocytes against palmitate-induced apoptosis. Thus, our study suggests an important role of miR-195 in apoptosis and reveals a novel signal mechanism by which palmitate induces apoptosis in cardiomyocytes.

miR-195 has been suggested to play an important role in the development of cardiac hypertrophy and heart failure.[21] In the present study, we showed that miR-195 also contributed to apoptosis in cardiomyocytes. In response to palmitate, inhibition of miR-195 prevented apoptosis in cardiomyocytes. On the other hand, the miR-195 mimic enhanced apoptosis. Because the loss of cardiomyocytes significantly contributes to heart diseases and heart failure, the finding that miR-195 mediates palmitate-induced cardiomyocyte apoptosis suggests that miR-195 may be an important factor in lipotoxic cardiomyopathy and may represent a new therapeutic target; however, this possibility needs to be examined in *in vivo* animal models in future studies.

miRNAs exert their actions by targeting specific mRNAs and inhibiting their protein expressions.[7,8,15,19] In this regard, we demonstrated that Sirt1 is a direct target of miR-195 in palmitate-induced cardiomyocytes. Several lines of evidences support this conclusion. First, computational prediction of targets identified two putative-

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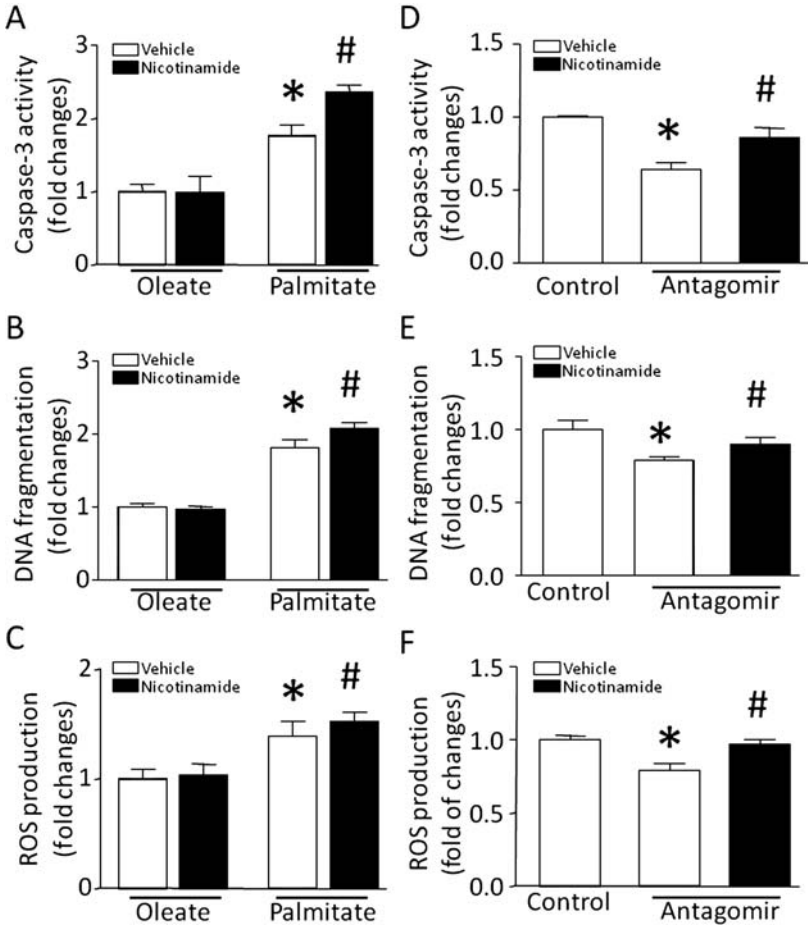

Figure 5 Effects of nicotinamide on apoptosis and ROS production. (*A*–*C*) Cardiomyocytes were incubated with palmitate or oleate in the presence of nicotinamide (50 μM) or vehicle for 24 h, and caspase-3 (*A*), DNA fragmentation (*B*), and intracellular ROS (*C*) were measured in cardiomyocytes. (*D*–*F*) Cardiomyocytes were transfected with miR-195 antagomir or a scrambled oligonucleotide as a control. Twenty-four hours later, these cells were incubated with palmitate in the presence of nicotinamide or vehicle for another 24 h. Caspase-3 (*D*), DNA fragmentation (*E*), and intracellular ROS (*F*) were measured in cardiomyocytes. Data are MEAN ± SD from five different cell cultures. *$P < 0.05$ vs. oleate in vehicle or control; #$P < 0.05$ vs. palmitate or antagomir in vehicle.

binding sites on the 3′UTR of Sirt1 mRNA. This was experimentally validated by utilizing a luciferase reporter activity assay which showed that the miR-195 mimic decreased the luciferase activity of the reporter vector containing the miR-195 response elements; in contrast, the miR-195 mimic had a minimal effect on a reporter vector with mutated miR-195 response elements. Second, palmitate mirrored the effect of miR-195 mimic on the luciferase activity of the reporter vector containing the miR-195 response elements or mutated miR-195 response elements in cardiomyocytes. Third, miR-195 induction correlated with a reduction in Sirt1 protein in palmitate-stimulated cardiomyocytes. More importantly, inhibition of miR-195 up-regulated, whereas the miR-195 mimic down-regulated Sirt1 protein in cardiomyocytes. These data demonstrate a functional significance of miR-195 induction in inhibition of Sirt1 protein expression in response to palmitate.

Whereas Sirt1 is an important regulator of energy homeostasis in response to nutrient availability and associated with increased longevity, recent studies have suggested that Sirt1 is an endogenous apoptosis inhibitor in cardiomyocytes.[22–24,38–40] In agreement with the latter findings, we showed that down-regulation of Sirt1 correlated with apoptosis in palmitate-induced cardiomyocytes. To support the view that Sirt1 prevents apoptosis, we demonstrated that pharmacological activation of Sirt1 reduced apoptosis, whereas inhibition of Sirt1 enhanced apoptosis in response to palmitate. More importantly, inhibition of Sirt1 with nicotinamide attenuated the protective effects of miR-195 antagomir in palmitate-induced apoptosis. Thus, our data suggest that the role of miR-195 is mediated, at least partly, through inhibition of Sirt1 in palmitate-induced apoptosis. Since each miRNA targets multiple mRNAs, it is possible that induction of miR-195 may also modulate other apoptotic genes in cardiomyocytes. In this regard, we showed that Bcl-2 was another target of miR-195 in regulating palmitate-induced apoptosis, which is consistent with a recent report.[37] In addition, miR-195 has been also suggested to block the G1/S transition by suppressing the expression of cyclin D1, CDK6, and E2F3,[41] which may be involved in apoptosis. However, this will require further studies for clarification.

In this study, ROS production was increased in palmitate-stimulated cardiomyocytes, which is in agreement with previous studies.[33] We

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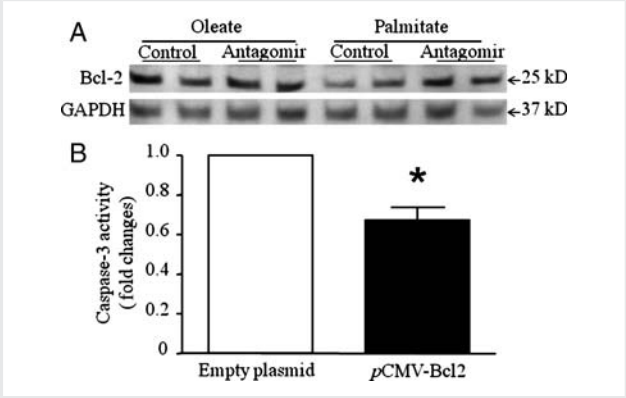

Figure 6 Bcl-2 expression and its role in caspase-3 activation. Cardiomyocytes were transfected with miR-195 antagomir or a scrambled oligonucleotide as a control. Twenty-four hours later, these cells were incubated with palmitate or oleate for another 24 h. Western blot was performed to determine Bcl-2 protein in cardiomyocytes. A is a representative western blot for Bcl-2 protein from three different cell cultures (duplicate in each group). (*B*) H9C2 cells were transfected with *p*CMV-Bcl2 or an empty plasmid as a control. Twenty-four hours later, these cells were incubated with palmitate for another 24 h and caspase-3 activity was then measured. Data are MEAN ± SD from three different cell cultures. *$P < 0.05$ vs. Empty plasmid.

further demonstrated that miR-195 promoted ROS production in palmitate-stimulated cardiomyocytes. The role of miR-195 in ROS production may be mediated through down-regulation of Sirt1 in cardiomyocytes since inhibition of Sirt1 increased, whereas activation of Sirt1 inhibited ROS production. Previous studies have also demonstrated that Sirt1 activation prevents ROS production presumably by inducing superoxide dismutase 2 and catalase expression.[38,42,43] ROS production is a contributing factor to apoptosis in cardiomyocytes.[44] Consistently, we showed that scavenging ROS by incubation with NAC dose-dependently inhibited apoptosis in palmitate-stimulated cardiomyocytes, supporting an important role of ROS production in palmitate-induced apoptosis. To further support the involvement of ROS production, we demonstrated that blocking mitochondrial ROS by the mitochondrial targeted antioxidant peptide prevented apoptosis. This result also suggests that mitochondrial ROS production may be one of the mechanisms by which palmitate induces apoptosis in cardiomyocytes. In non-cardiomyocytes, including neutrophils, fibroblasts, endothelial cells, pancreatic beta cells, and hepatic cells, an increase in ROS production has been demonstrated to account for apoptosis in response to palmitate.[45–48] However, these previous demonstrations and our present result are apparently in disagreement with a previous report that demonstrated that palmitate-induced apoptosis in neonatal rat cardiomyocytes was not dependent on ROS production.[49] It is currently unknown what causes this discrepancy. Given the fact that palmitate-induced cell death was inhibited by NAC in rat H9C2 cardiac muscle cells (data not shown), this discrepancy may be not due to cells from different species used, as the present study used neonatal mouse cardiomyocytes. Nevertheless, our data support the view that down-regulation of Sirt1 promotes apoptosis through ROS production in palmitate-stimulated cardiomyocytes. Since the anti-apoptotic role of Sirt1 has also been associated with p53, Ku70 and forkhead transcription factors,[50–52] the present study could not exclude the involvement of these pathways in palmitate-induced apoptosis, which merits future studies.

It is important to point out that the purity of cultured cardiomyocytes was >90% and palmitate could not induce apoptosis in cultured cardiac fibroblast cells (main cell type of contaminated non-cardiomyocytes). Thus, the interference from contaminated non-cardiomyocytes (<10%) was negligible. It is also worthwhile to mention that neither miR-195 mimic nor antagomir had any evident effects on basal ROS production and apoptosis in cardiomyocytes while overexpression of miR-195 induced cardiac hypertrophy in a transgenic mouse model.[21] This discrepancy may be due to (i) the levels of miR-195 mimic or antagomir in cardiomyocytes were lower compared with those in the transgenic mice overexpressing miR-195; (ii) the miR-195 mimic or antagomir had only temporary effects in cardiomyocytes, whereas the effects of transgenic miR-195 overexpression were sustained for a long term. In fact, inhibition or overexpression of other miRs, for example miR-199a, has no effects on basal apoptosis but significantly affects stress-induced apoptosis in cardiomyocytes.[19] Finally, although Sirt1 has been shown to inhibit ROS production and apoptosis,[43] we did not see any effects of Sirt1 knockdown on basal ROS and apoptosis in cardiomyocytes. This suggests that Sirt1 may only suppress induced ROS production and apoptosis in cardiomyocytes under stresses. Indeed, transgenic overexpression of Sirt1 did not alter basal oxidative stress but inhibits stress-induced oxidative stress in the heart.[38] Similarly, cardiac-specific knockout of Sirt1 did not exhibit any pathological phenotype at 3 months of age.[53]

In summary, this study demonstrates a novel pro-apoptotic role of miR-195 in cardiomyocytes and verifies Sirt1 as a direct target of miR-195. The effect of miR-195 induction on apoptosis is mediated through down-regulation of Sirt1 and Bcl-2, and ROS production. Thus, miR-195 may be a new therapeutic target for lipotoxic cardiomyopathy.

Conflict of interest: none declared.

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