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Journal of Cell Science 124, 3187

doi:10.1242/jcs.098830

Retraction of: *J. Cell Sci*. **120**, 3045–3052.

After it was brought to our attention by the *Journal of Cell Science*, careful examination of the above paper published in the journal in 2007 highlighted some errors we made related to the re-use of our previously published western blot bands in parts A, B and C of Figure 3, and duplication of western blot bands in Figures 3A and 4A. The misuse and re-use of western blot bands violated the editorial policy of *Journal of Cell Science*, and so we must retract this article.

The errors originated in Dr Zhiguo Wang's laboratory at the Montreal Heart Institute. Dr Wang takes full responsibility and apologises to the editors and readership of *Journal of Cell Science* for any inconvenience caused and any negative impact this might have on the journal.

The muscle-specific microRNAs *miR-1* and *miR-133* produce opposing effects on apoptosis by targeting HSP60, HSP70 and caspase-9 in cardiomyocytes

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Accepted 26 June 2007
Journal of Cell Science 120, 3045-3052 Published by The Company of Biologists 2007
doi:10.1242/jcs.010728

Summary

The microRNAs *miR-1* and *miR-133* are preferentially expressed in cardiac and skeletal muscles and have been shown to regulate differentiation and proliferation of these cells. We report here a novel aspect of cellular function of *miR-1* and *miR-133* regulation of cardiomyocyte apoptosis. *miR-1* and *miR-133* produced opposing effects on apoptosis, induced by oxidative stress in H9c2 rat ventricular cells, with *miR-1* being pro-apoptotic and *miR-133* being anti-apoptotic. *miR-1* level was significantly increased in response to oxidative stress. We identified single target sites for *miR-1* only, in the 3′-untranslated regions of the HSP60 and HSP70 genes, and multiple putative target sites for *miR-133* throughout the sequence of the caspase-9 gene. *miR-1* reduced the levels of HSP60 and HSP70 proteins without changing their transcript levels, whereas *miR-133* did not affect HSP60 and HSP70 expression at all. By contrast, *miR-133* repressed caspase-9 expression at both the protein and mRNA levels. The post-transcriptional repression of HSP60 and HSP70 and caspase-9 was further confirmed by luciferase reporter experiments. Our results indicate that *miR-1* and *miR-133* are involved in regulating cell fate with increased *miR-1* and/or decreased *miR-133* levels favoring apoptosis and decreased *miR-1* and/or *miR-133* levels favoring survival. Post-transcriptional repression of HSP60 and HSP70 by *miR-1* and of caspase-9 by *miR-133* contributes significantly to their opposing actions.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/17/3045/DC1

Key words: *miR-1*, *miR-133*, Apoptosis, HSP60, HSP70, Caspase-9

Introduction

MicroRNAs (miRNAs) are endogenous ~22 nucleotide (nt) non-coding RNAs that anneal to inexactly complementary sequences in the 3′-UTRs of target mRNAs of protein-coding genes to specify translational repression or/and mRNA cleavage (Ambros, 2004; Jackson and Standart, 2007; Meister and Tuschl, 2004; Lim et al., 2005). Having generated a tremendous amount of excitement about miRNAs in many areas of biology, research over the past five years has put miRNAs at centre stage. However, in spite of our ability to identify miRNAs, regulatory targets have not been established or even confidently predicted for any of the vertebrate miRNAs, which has hampered progress toward elucidating the functions of miRNAs. Our current understanding of the functions of miRNAs primarily relies on their tissue-specific or developmental stage-specific expression patterns as well as their evolutionary conservation and thus is limited to developmental regulation and oncogenesis (Ambros, 2004; Hwang and Mendell, 2006; Hammond, 2006), and much less is known about their role in other biological processes.

Among the known miRNAs, *miR-1* and *miR-133* are believed to be specifically expressed in adult cardiac and skeletal muscle tissues (Zhao et al., 2005; Chen et al., 2006; Rao et al., 2006; Kwon et al., 2005). Both of them have been found in most animal species, from *Drosophila* to human, indicating that they are evolutionally conserved. Increasing expression was found in neonatal hearts and skeletal muscle, and substantially higher levels are maintained in adult muscular tissues. Authors of a recent study (Chen et al., 2006) have proposed a model in which *miR-1* and *miR-133* regulate myogenesis by controlling distinct aspects of the differentiation process; *miR-1* promotes myogenic differentiation and *miR-133* enhances myoblast proliferation. One of the questions we asked is whether *miR-1* and *miR-133* are involved in apoptotic cell death under pathological conditions relevant to human cardiac disease. This study was designed to shed light on this issue.

Results

miR-1 and *miR-133* produce opposing regulations on cardiomyocyte apoptosis

H9c2 cells (rat embryonic ventricular cell line) were divided into five groups: control, wild-type (WT) *miR-1*, mutant (MT)

miR-1, WT *miR-133* and MT *miR-133* groups, and they were all subjected to transfection procedures. Apoptotic cell death was verified by TUNEL of chromosomal cleavage (Fig. 1A) and enzyme-linked immunosorbent assay (ELISA) quantification of DNA fragmentation (by measuring optical density). Overexpression of *miR-1* induced significant apoptotic cell death; DNA fragmentation increased by ~15 fold (Fig. 1B). By comparison, overexpression of *miR-133* did not cause H9c2 cell death. The apoptosis-inducing effect was not seen in cells transfected with MT *miR-1*. Noticeably, co-transfection of *miR-1* and *miR-133* largely prevented the apoptosis caused by *miR-1* alone, indicating an anti-apoptotic effect of *miR-133*.

Incubation of H9c2 cells with H_2O_2 (ranging from 0 to 600 μM) produced concentration-dependent cell death, as determined by ELISA. The mean IC_{50} value for DNA fragmentation induction by H_2O_2 was 65.5±5.2 μM (n=5 batches of cells) under our experimental conditions. Furthermore, overexpression of WT *miR-1* promoted H_2O_2-induced apoptosis, as indicated by ~60% reduction of IC_{50} value (Fig. 1C). In sharp contrast, overexpression of WT *miR-133* produced a cytoprotective effect against

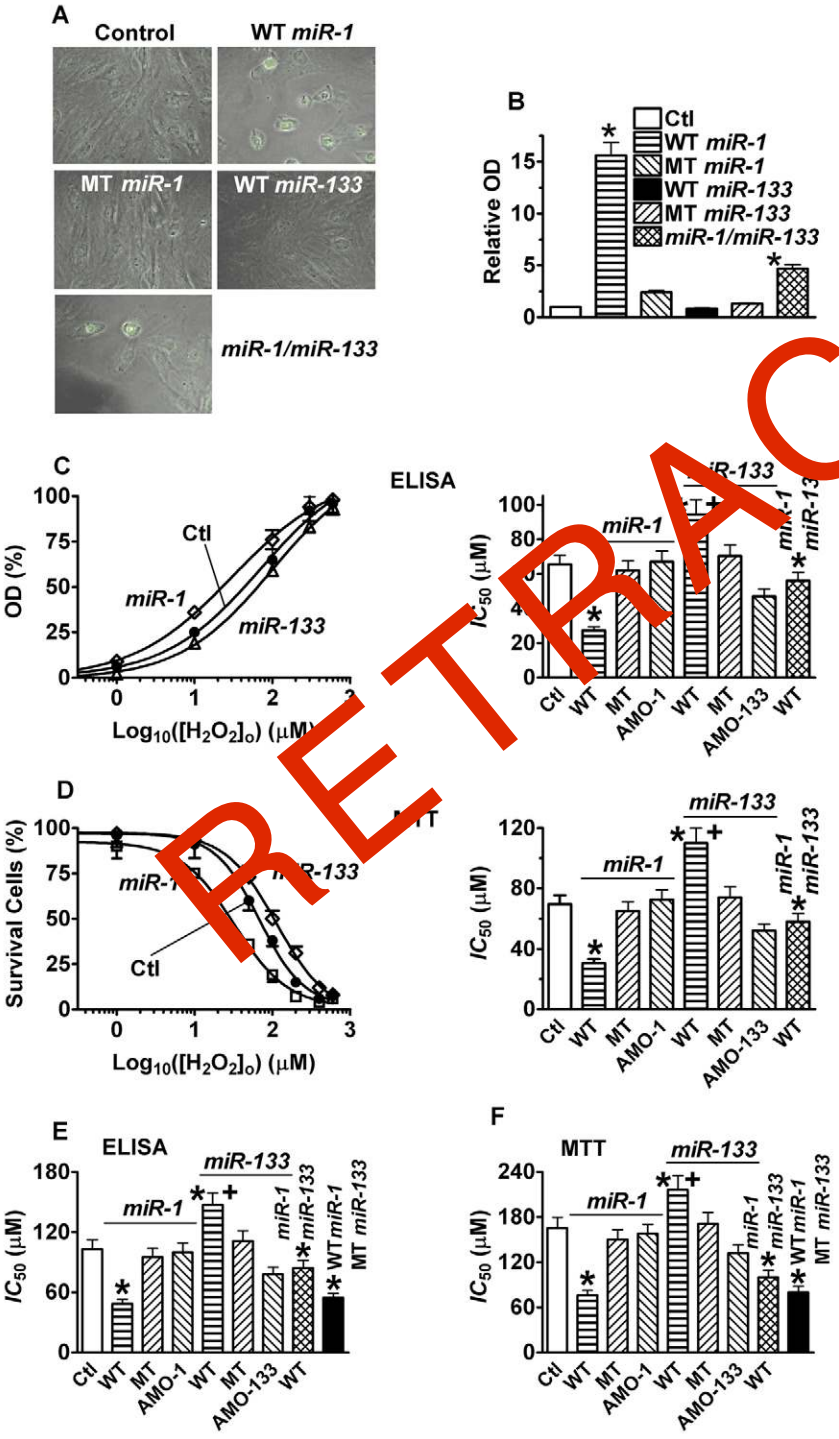

Fig. 1. *MiR-1* and *miR-133* produce opposing effects on cardiomyocyte apoptosis. (A) Examples of TUNEL staining showing the effects of various constructs, as labeled, on chromosomal condensation. (B) Pro-apoptotic effect of *miR-1* and anti-apoptotic effect of *miR-133*, determined by ELISA quantification of DNA fragmentation expressed as optical density (OD) values. Ctl, control; WT, wild-type; MT, mutant. (C) Promoting effect of *miR-1* and antagonizing effect of *miR-133* on H_2O_2-induced apoptosis determined by ELISA. The lines represent fits to the Hill equation to define the concentration of H_2O_2 ($[H_2O_2]_o$) for half-maximum cell death (IC_{50}). AMO-1 and AMO-133 are anti-miRNA oligonucleotides specific for *miR-1* and *miR-133*, respectively (same below). (D) Antagonizing effect of *miR-1* and promoting effect of *miR-133* on cell survival, as determined by MTT, in the presence of various concentrations of H_2O_2. The symbols are experimental data points and the lines represent fits to the Hill equation. Data in A-D were obtained from the rat ventricular cell line H9c2 cells. (E) Promoting effect of *miR-1* and antagonizing effect of *miR-133* on apoptosis, as determined by ELISA, induced by various concentrations of H_2O_2 in isolated neonatal rat ventricular myocytes (same below). Note that co-transfection of wild-type (WT) *miR-1* and WT *miR-133* abrogated the effects seen with WT *miR-1* alone, whereas co-transfection of *miR-1* and MT *miR-133* produced effects similar to transfection of WT *miR-1* alone. (F) Antagonizing effect of *miR-1* and promoting effect of *miR-133* on cell survival, as determined by MTT, in the presence of various concentrations of H_2O_2. Data are presented as means ± s.e.m. (n=8, 6, 6, 8 and 8, respectively). *P<0.05 versus (vs) Ctl; +P<0.05 vs WT *miR-1*.

H_2O_2-induced apoptosis; the IC_{50} was increased by ~40%. Consistently, overexpression of *miR-133* markedly increased cell viability, whereas that of *miR-1* did the opposite, as determined by MTT assay (Fig. 1D). These effects were not seen when MT *miR-1* or MT *miR-133* was transfected. To further verify the opposing effect of *miR-1* and *miR-133*, we performed reciprocal experiments wherein we transfected H9c2 cells with 2′-*O*-methyl antisense inhibitory oligonucleotides (AMOs) against *miR-1* (AMO-1) or *miR-133* (AMO-133) (Krutzfeldt et al., 2005; Cheng et al., 2005). AMO-1 reduced H_2O_2-induced apoptosis, whereas AMO-133 facilitated it (Fig. 1C,D). Most strikingly, co-transfection of WT *miR-1* and WT *miR-133* failed to alter H_2O_2-induced apoptosis, indicating that they counteract each other.

To confirm the above results obtained with the H9c2 cell line, the same experiments were conducted in neonatal rat ventricular myocytes, which are known to be terminally differentiated cardiomyocytes. Quantitatively the same results were obtained with these cells (Fig. 1E,F).

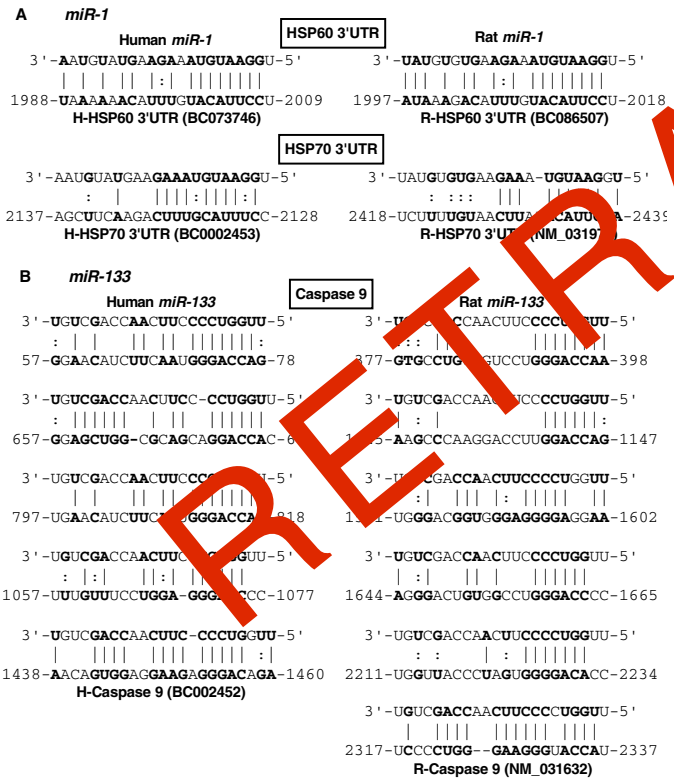

Fig. 2. The sequences showing the unique sites of miRNA::mRNA complementarity between *miR-1* and HSP60 or HSP70, and between *miR-133* and caspase-9 (Casp9) for both human (H) and rat (R) genes. The matched base pairs are bold and connected by a vertical line and the G:U/U:G wobble is indicated by bold letters connected by dots. The GenBank accession numbers of the genes are indicated in the brackets and the positions of the target sites are numbered. Note that there is only a single target site for *miR-1* in HSP60 or HSP70 and the complementarity is limited to the 3′UTRs of these genes, whereas there are multiple target sites in Casp9 and the sites are distributed throughout the whole mRNA sequence.

Post-transcriptional repression of HSP60 and HSP70 by *miR-1* and of Casp9 by *miR-133*

The above results suggest that *miR-1* is pro-apoptotic whereas *miR-133* is anti-apoptotic and these opposing actions are probably due to regulation of distinct apoptotic and survival factors by these miRNAs. To test this notion, we first used a computational and bioinformatics-based approach to predict the putative targets related to apoptosis versus survival using TargetScan hosted by the Wellcome Trust Sanger Institute (Griffiths-Jones, 2004). In this way, we identified two important candidate targets for *miR-1*: HSP60 and HSP70 (heat shock proteins). We also identified several targets sites for *miR-133* in the caspase-9 (Casp9) gene by complementarity (Fig. 2).

To verify that HSP60, HSP70 and Casp9 are indeed the cognate targets of *miR-1* and *miR-133*, respectively, for post-transcriptional repression, we took the following approaches. We first determined the effects of the two miRNAs on protein levels, by western blotting and caspase activity assays. *miR-1* remarkably reduced the levels of HSP60 proteins by ~73% in H9c2 cells both in the absence and presence of H_2O_2 (Fig. 3A). HSP70 protein level was similarly reduced, albeit to a less extent (~53%; Fig. 3B). Co-application of *miR-1* and AMO-1 almost abolished the effects. By comparison, *miR-133* decreased total Casp9 protein level by ~89% (Fig. 3C), an effect eliminated by co-transfection with AMO-133. When transfected with MT *miR-1* and MT *miR-133*, decreases in HSP60, HSP70 and Casp9 were hardly seen. Since reduction of Casp9 protein level may well result in reduction of Casp9 activation, Casp9 activities were determined. As shown in Fig. 3D, *miR-133* diminished basal Casp9 activity and prevented H_2O_2-induced increase in Casp9 activity. These effects of *miR-133* were antagonized by AMO-1. Moreover, application of AMOs alone increased the levels of HSP60, HSP70 and Casp9 in H9c2 cells (Fig. 3E), indicating the roles of basal *miR-1* and *miR-133* in cardiac cells.

We subsequently investigated the effects of the miRNAs on mRNA levels of HSP60, HSP70 and Casp9 in H9c2 cells. The mRNA levels of HSP60, HSP70 were unaffected by *miR-1*, indicating that *miR-1* and *miR-133* do not affect their mRNA stability. Casp9 mRNA was reduced by *miR-133* (Fig. 3F).

Effects of *miR-1* and *miR-133* on caspase-3 (Casp3) activities

Casp3 genes do not contain any domains bearing putative target sites for *miR-1* or *miR-133*. Thus, *miR-1* or *miR-133* is not expected to alter Casp3 protein levels. This was indeed verified by our experiments using western blot analysis with the antibody against the total Casp3 protein (Fig. 4A). However, *miR-1* and *miR-133* may indirectly affect Casp3 activities because these miRNAs repress HSP60, HSP70 and Casp9, which could in turn alter Casp3 activities (but not total protein levels). To test this notion, we determined Casp3 activities in H9c2 cells with or without H_2O_2 treatments, *miR-1*, *miR-133* and their AMOs. Transfection of cells with *miR-1* or challenge of cells with H_2O_2 both robustly increased Casp3 activities and the effects were abolished by co-application of AMO-1. By contrast, transfection of cells with *miR-133* diminished Casp3 activity, an effect prevented by co-application of AMO-133. Moreover, H_2O_2 enhanced Casp3 activity and co-application with AMO-133 further

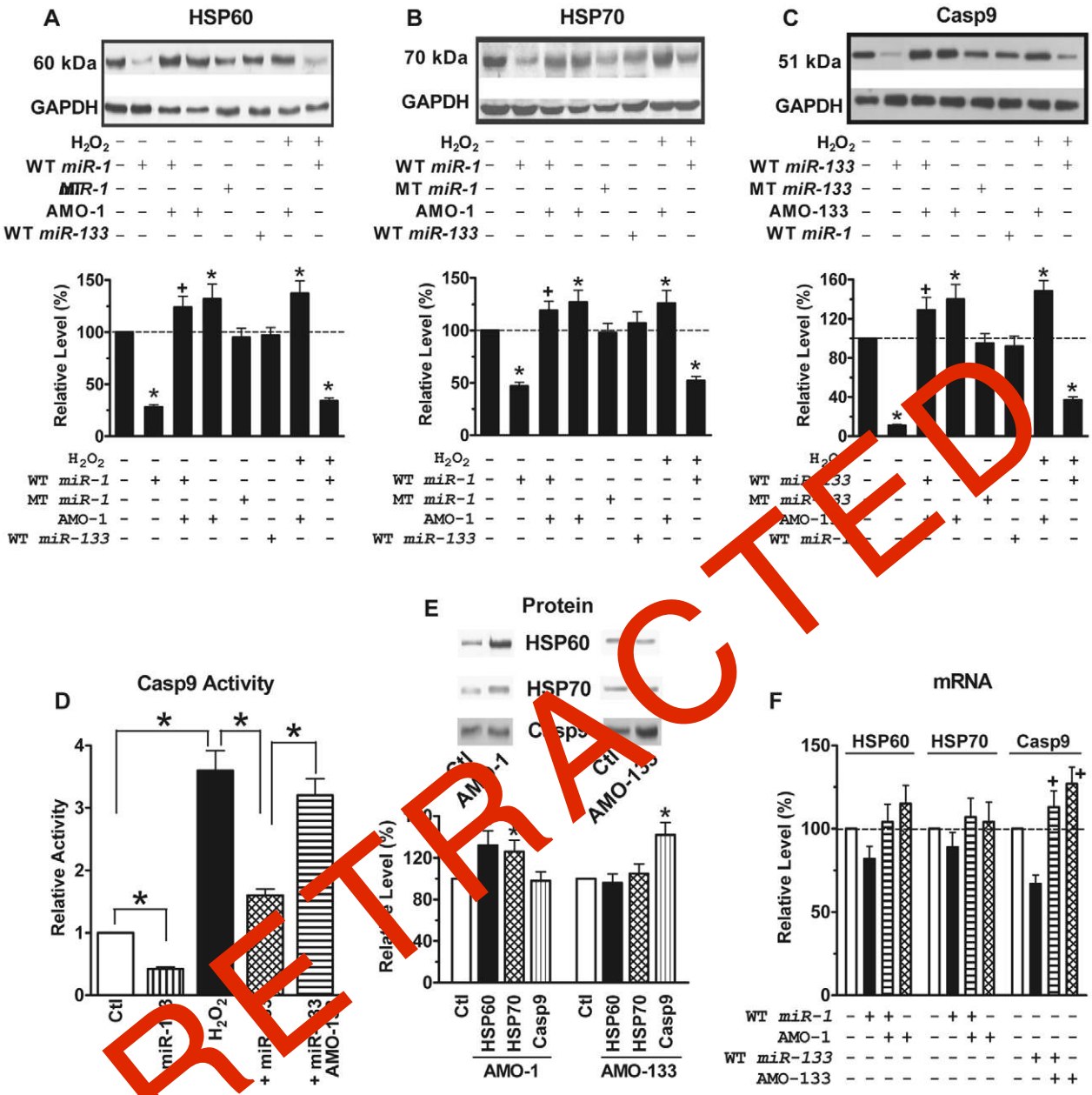

Fig. 3. Post-transcriptional repression of HSP60 and HSD70 by *miR-1* and caspase-9 (Casp9) by *miR-133*. (A-C) Western blot analysis of HSP60, HSP70 and Casp9 (total) under various conditions with protein samples from H9c2 cells. The bar charts in the lower panels represent the densitometric measurements of western blots of HSP60, HSP70 and Casp9 expression. (D) Effects of *miR-133* on Casp9 activity. (E) Effects of AMO-1 and AMO-133, respectively, on protein levels of HSP60, HSP70 and Casp9 in H9c2 cells. (F) Effects of *miR-1* and *miR-133* on mRNA levels of HSP60, HSP70 and Casp9 in H9c2, as determined by real-time RT-PCR. Data presented as means ± s.e.m. (n=8, 6, 6, 5, 6, and 6, for A-F, respectively). *P<0.05 vs Ctl; ^{+}P<0.05 vs WT *miR-1* or WT *miR-133*.

increased Casp3 activity (Fig. 4B), in line with the notion that *miR-133* diminishes Casp3 activation as a result of repression of Casp9.

Verification of interactions between *miR1*, *miR-133* and their target genes

We placed the 3′UTRs of HSP60 and HSP70, or the full-length cDNA of Casp9 into the 3′UTR of a luciferase reporter plasmid to construct chimeric vectors. Co-transfection of the chimeric constructs with *miR-1* or *miR-133* (Fig. 5) into HEK293 cells, consistently resulted in smaller luciferase activity relative to transfection of the chimeric plasmid alone. Co-application of *miR-1* or *miR-133* with their respective AMOs eliminated the silencing effects. A mutated target sequences of HSP60 or HSP70 fused to the 3′UTR of luciferase was not responsive to *miR-1* or *miR-133*, suggesting specificity of the repression effect. Furthermore, MT *miR-1* or MT *miR-133* had no effect on the WT target sequences, but could efficiently repress luciferase activities with the constructs containing the MT 3′UTRs of HSP60 or HSP70 complementary to MT *miR-1*.

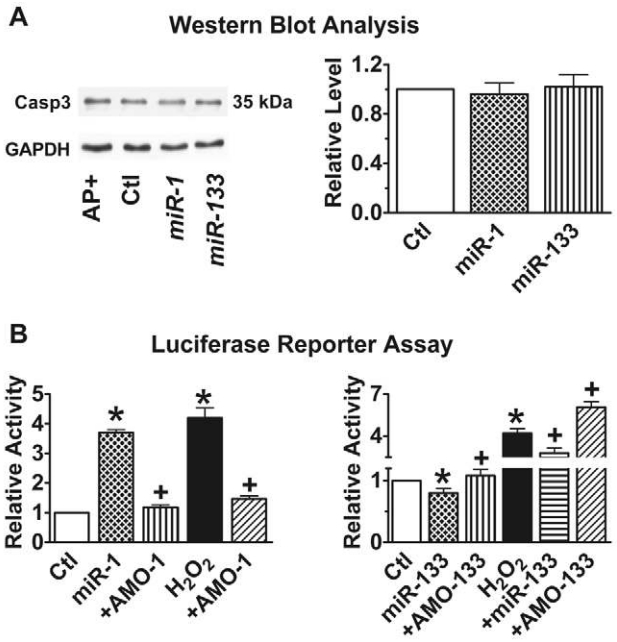

Fig. 4. Effects of *miR-1* and *miR-133* on caspase 3 (Casp3) protein levels and activities in H9c2 cells. (A) Immunoblotting analysis of Casp3 protein levels with and without *miR-1* or *miR-133* treatment. The antibody against the total Casp3 recognized the 35 kDa band representing Casp3. AP+: antibody pretreated with its antigenic peptide; Ctl: cells treated with Lipofectamine 2000 only; *miR-1* and *miR-133*: cells transfected with *miR-1* and *miR-133*, respectively and with Lipofectamine 2000. n=8 experiments for each group. (B) Regulation of Casp3 activity by *miR-1* and *miR-133*. Transfection was performed with Lipofectamine 2000 and measurements were made 24 hours after transfection. $^{*}P<0.0$ vs Ctl; $^{+}P<0.05$ vs *miR-1* alone or *miR-133* alone or H_2O_2 alone; n=5 for each group.

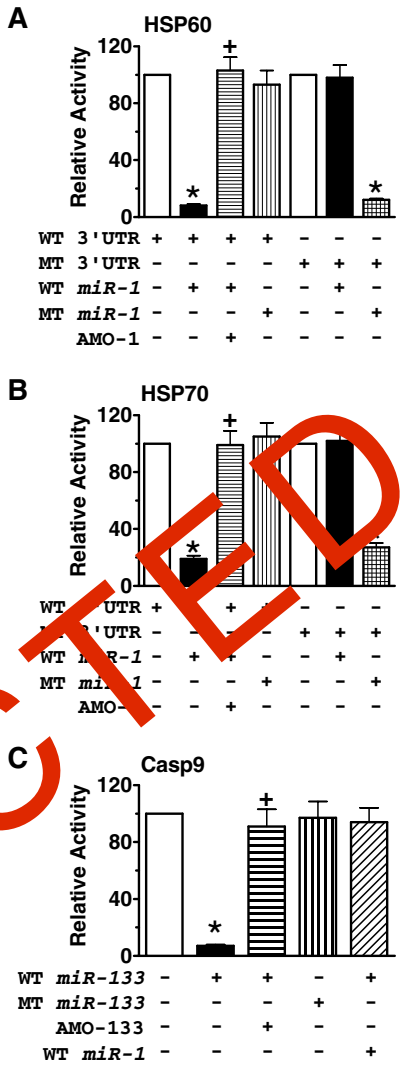

Fig. 5. Verification of HSP60 (A), HSP70 (B) and Casp9 (C) as cognate targets of *miR-1* and *miR-133*, respectively, for post-transcriptional repression. Data on luciferase reporter activities show the interaction between *miR-1* and HSP60 and HSP70 3′UTRs and between *miR-133* and Casp9 mRNA. WT, wild type; MT, mutant, AMO-1 and AMO-133, *miR-1*- and *miR-133*-specific antisense inhibitors, respectively (see Fig. 2). Shown are means ± s.e.m. (n=5 batches of cells for each bar in A-C). $^{*}P<0.05$ vs Ctl; $^{+}P<0.05$ vs WT *miR-1* or WT *miR-133*.

We then used *miR-1* and *miR-133* standards in which the complementary sequences of *miR-1* and *miR-133* were cloned downstream of the luciferase gene in the pMIR-REPORT plasmid (Chen et al., 2006; Krützfeldt et al., 2005). With these constructs, we were able to control the uptake and activities of transfected miRNAs. Real-time RT-PCR analyses on the effects of AMO-1 and AMO-133 on *miR-1* and *miR-133* levels in H9c2 cells, to verify the efficacy and specificities against the exogenous miRNAs, have been reported in our previous study (Yang et al., 2007; Luo et al., 2007).

Successful delivery of *miR-1* or *miR-133* and AMO-1 or AMO-133 into the cells was further verified by comparing the *miR-1* or *miR-133* levels before and 48 hours after transfection of the constructs in cultured neonatal ventricular myocytes. As shown in Fig. 6A, transfection resulted in approximately three- to fourfold increases in *miR-1* and *miR-133* levels. For control purpose, transfection of *miR-1* or *miR-133* did not significantly alter the level of *miR-133* or *miR-1*. It should be mentioned that the *miR-1* and *miR-133* levels are dynamic with transfection. Our data were collected at a specific time, 48 hours after transfection (because all our measurements were performed at this time) and the levels do not apply to other times. Coincidently, incubation of cells with H_2O_2 (150 μM) caused an approx. threefold elevation of *miR-1* (Fig. 6B). By comparison, H_2O_2 induced only 70% increase in *miR-13*. The results are consistent with the fact that H_2O_2 induces apoptosis.

The H9c2 rat ventricular cell line and the HEK293 human embryonic kidney cell line were used in our study for different specific objectives. The former was used for experiments involving endogenous *miR*-1 or *miR-133*, such as the experiments involving application of AMO-1 alone, and the latter was used for experiments involving only exogenously delivered *miR-1* or *miR-133* by transfection, such as the luciferase reporter gene experiments. We quantified the levels of *miR-1* and *miR-133* in these cell lines as well as in the A549 human lung cancer cell line. Our data confirmed that H9c2 expresses endogenous *miR-1* and *miR-133,* which are known to be muscle-specific, whereas the non-muscle cells HEK293

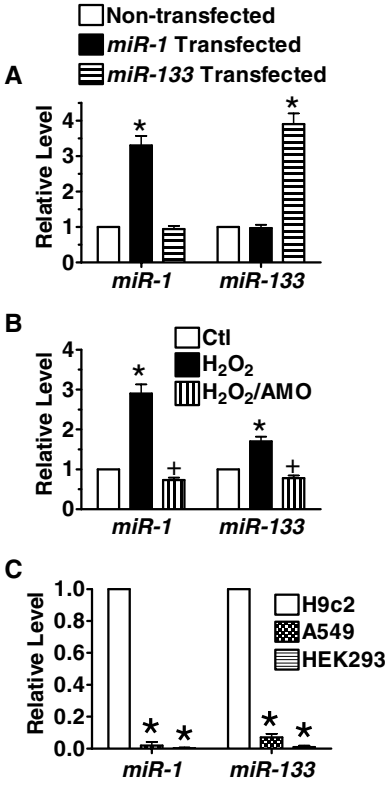

Fig. 6. Comparison of *miR-1* and *miR-133* expression levels under various conditions, measured by real-time RT-PCR. (A) *miR-1* and *miR-133* levels without and with transfection of exogenous *miR-1* and *miR-133*. The data are averaged from three batches of H9c2 cells. *P<0.05 vs non-transfected cells. (B) Enhanced expression of *miR-1* and *miR-133* induced by H_2O_2 (150 μM) in H9c2 cells (n=5 batches of cells for each group). *P<0.05 vs Ctl; ^+P<0.05 vs *miR-1* or *miR-133*. (C) Comparison of *miR-1* and *miR-133* expression levels in various cell lines indicated. H9c2, rat ventricular cell line (n=6 batches of cells); A549, human lung cancer cell line (n=7 batches of cells); HEK293, human embryonic kidney cell line (n=7 batches of cells). *MiR-1* and *miR-133* levels are expressed as relative levels by normalizing to the values obtained from H9c2 cells. *P<0.05 vs H9c2.

and A549 express only minimal levels (~1/100-1/1000 of H9c2) of endogenous *miR-1* and *miR-133* (Fig. 6C).

Discussion

The study reported by Chen et al. (Chen et al., 2006) revealed that *miR-1* and *miR-133* are clustered on the same chromosomal loci and transcribed together as a single transcript which becomes two independent, mature miRNAs with distinct biological functions. One important characteristic of *miR-1* and *miR-133* regulation of cardiomyocyte apoptosis, revealed in this study, is that they produce opposing effects; *miR-1* is pro-apoptotic whereas *miR-133* is anti-apoptotic. This suggests that the relative levels of *miR-1* and *miR-133* is more important than their absolute levels, to determining the fate (apoptosis and survival) of cardiac cells. This notion is supported by the fact that co-transfection of *miR-1* and *miR-133* failed to induce apoptosis or to affect oxidative stress-induced apoptosis. In addition, in the presence of oxidative stress to induce apoptosis, both *miR-1* and *miR-133* levels were increased relative to those in cells under normal conditions, but the increase in *miR-1* predominantly overweighed that in *miR-133*, favoring the occurrence of apoptosis. However, at this stage the notion is merely a speculation that needs further studies to verify. Moreover, it should also be noted that this study merely provided indirect evidence for the interactions between *miR-1*, *miR-133* and their target genes, and more rigorous experimentation is required to fully establish the relationships.

Mitochondrial death pathway is one of the major mechanisms for apoptosis, which involves selective disruption of the outer membrane as a result of mitochondrial matrix hyperpolarization and/or matrix swelling, pore formation by proteins such as Bax and Bcl-xS, or ra[illegible]s of $\Delta\Psi$ following permeability transition (Latchman, 2001; Gupta and Knowlton, 2005). HSPs are expressed both constitutively (cognate proteins) and under stressful conditions (inducible forms), with constitutive expression being most prominent in mammalian tissues. HSPs are primarily anti-apoptotic and different HSPs have been shown to inhibit the mitochondrial death pathway at different points. HSP60 in the heart has key anti-apoptotic functions because of its ability to form complexes with Bax, Bak and Bcl-xS (Lin et al., 2001; Kirchhoff et al., 2002; Shan et al., 2003; Marber et al., 1995), but not with Bcl-2. Binding of HSP60 in the normal cardiac cells prevents Bax from oligomerizing and inserting into the mitochondrial membrane. Reduction of HSP60 is associated with an overall decrease in Bcl-2 along with an increase in Bax and Bak and is sufficient to precipitate apoptosis (Lin et al., 2001; Kirchhoff et al., 2002; Shan et al., 2003; Marber et al., 1995). HSP70 exerts its anti-apoptotic effect by preventing oligomerized Apaf-1 from recruiting pro-Casp9 (Latchman, 2001; Marber et al., 1995). HSP70 can also inhibit apoptosis in a caspase-independent manner by inhibiting the c-Jun N-terminal kinase (JNK kinase). However, Casp9 is a critical regulator of mitochondria-mediated apoptosis; it forms a multimeric complex with cytochrome c and Apaf-1 to activate downstream caspases such as caspase-3 leading to apoptotic cell death (Han et al., 2006; Bialik et al., 1999; Kannan and Jain, 2000). The data in the present study showing repression of HSP60 and HSP70 by *miR-1* and Casp9 by *miR-133* and the opposing actions of these two miRNAs on apoptosis are in line with these previous findings.

Our data demonstrated silencing of HSP60 and HSP70 by *miR-1* only at the protein level, and knockdown of Casp9 by *miR-133* at both protein and mRNA levels. Earlier, miRNAs were though to primarily repress their targets at the protein level without affecting mRNA stability (Meister and Tuschl, 2004; Lewis et al., 2003). Increasing evidence, however, indicate that miRNAs silence genes by multiple mechanisms including degrading their target mRNAs (Kannan and Jain, 2000; Nilsen, 2007; Pillai et al., 2007). Our observations seem to be in line with multiple mechanisms of the action. However, it is presently unclear what determines the exact mechanisms of miRNA actions.

Collectively, our study revealed a novel aspect of cellular functions of the muscle-specific miRNAs *miR-1* and *miR-133*, i.e. regulation of apoptosis and survival in cardiomyocytes. A unique feature of this regulation is the opposing actions with *miR-1* being pro-apoptotic and *miR-133* being anti-apoptotic,

suggesting a possible role of relative *miR-1* and *miR-133* levels in regulating the cell fate. Post-transcriptional repression of HSP60 and HSP70 by *miR-1* and of Casp9 by *miR-133* is probably one of the mechanisms underlying their regulation of apoptosis versus survival. Our present and previous studies revealed the pathological elevations of *miR-1* levels in cardiomyocytes in conditions favoring apoptosis (ischemia and oxidative stress). However, how these conditions lead to overexpression of *miR-1* remains unclear.

Materials and Methods

Cell culture

The cell lines used in this study were all purchased from American Type Culture Collection (ATCC, Manassas, VA). H9c2 (rat ventricular cell line) and HEK293 (human embryonic kidney cell line) were cultured in Dulbecco's Modified Eagle Medium (DMEM). The cultures were supplemented with 10% fetal bovine serum and 100 μg/ml penicillin/streptomycin.

Synthesis of miRNAs and sequences of miRNA inhibitors

miR-1 and *miR-133* and their respective mutant constructs were synthesized by Integrated DNA Technologies (IDT) (1). The sequences of *miR-1* and *miR-133* inhibitors (AMOs; anti-miRNA oligonucleotides) used in our studies are the exact antisense copies of their respective mature miRNA sequences: 3′-AAU**GU**A**U**G**AAGA**AA**UGUAAGGU**-5′ for human *miR-1* (GenBank acc. no.: HSM808714), 3′-AAU**GU**A**U**G**AAGA**AA**UGUAAGGU**-5′ for rat *miR-1* (GenBank ac. no.: DQ066650), and 3′-U**GU**C**G**ACCAACUUC**CCCUGGU**U-5′ for both human and rat *miR-133* [the sequences of *miR-133* are identical in human (HSM808714) and rat (RATNCRNAB)]. All the nucleotides in the AMOs (AMO-1 for *miR-1* and AMO-133 for *miR-133*) contain 2′-*O*-methyl modifications at every base and a 3′ C3-containing amino linker. The antagomers were also synthesized by IDT.

Construction of the chimeric miRNA binding site – luciferase reporter vectors and mutagenesis

To generate reporter vectors bearing miRNA-binding sites, we generated direct match *miR-1* and *miR-133* sites (synthesized by Invitrogen), respecti and the sequences around the putative target sites for these miRNAs i e 3′ UTRs of HSP60 and HSP70, and the full-length Casp9 mRNA (1). Thes serts we ned into the multiple cloning sites in the pMIR-REPORT™ l fer miR A expression reporter vector (Ambion, Inc.). The sense d antisens rands of the oligonucleotides were annealed by adding 2 μg of oligonucleoti to 46 μl of annealing solution (100 mM potassium acetate, mM pepes-KOH, p 7.4 and 2 mM magnesium acetate) and incubating at 90°C for 5 mi es and then at 37°C for 1 hour. The annealed oligonucleotides we digested with *Hin*III and *Spe*I and used to ligate into *Hin*dIII and *Spe*I sites.

Nucleotide-substitution mutation MT) w carried out using direct oligomer synthesis for *miR-1* and *miR-133*, a PC -based methods for the 3′ UTRs of HSP60 and HSP70 genes. T substitu n nucleo es were so designed to avoid producing new binding es for her miRN s potentially existing in HEK293 cells. All constructs were quencing rified. See g. S1 in supplementary material for details of the mutatio

Transfection of miRNAs and luciferase assay

After 24 hours starvation in serum-free medium, cells (1×10^5 per well) were transfected with 1 μg *miR-1*, *miR-133,* or other constructs, with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

For luciferase assay, cells were transfected with 1 μg PGL3-target DNA (firefly luciferase vector) and 0.1 μg PRL-TK (TK-driven *Renilla* luciferase expression vector) with Lipofectamine 2000. Luciferase activities were measured 48 hours after transfection with a dual luciferase reporter assay kit (Promega) on a luminometer (Lumat LB9507) (Yang et al., 2007; Luo et al., 2007).

Quantification of mRNA and miRNA levels

For quantification of HSP60, HSP70 and Casp9 transcripts, conventional real-time RT-PCR was carried out with total RNA samples extracted from H9c2 cells and neonatal rat ventricular cells 48 hours after transfection. TaqMan quantitative assay was performed with the expression level of GAPDH as an internal control.

The *mir*Vana™ qRT-PCR miRNA Detection Kit (Ambion) was used in conjunction with real-time PCR with SYBR Green I for quantification of *miR-1* and *miR-133* transcripts, as detailed elsewhere (Yang et al., 2007; Luo et al., 2007). Fold variations in expression of *miR-1* and *miR-133* between RNA samples were calculated after normalization to 5s rRNA.

Western blot analysis

The protein samples were extracted from H9c2 cells and cultured neonatal rat ventricular cells, with the procedures essentially the same as described in detail elsewhere (Han et al., 2001; Han et al., 2004a; Han et al., 2004b; Luo et al., 2007; Wang et al., 2002). Protein samples (~50 μg) were fractionated by SDS-PAGE (7.5-10% polyacrylamide gels). The primary antibodies against HSP60 (Stressgen Bioreagents, Ann Arbor, MI; rabbit polyclonal), HSP70 (Cell Signaling; rabbit polyclonal) and total caspase-3 and total caspase-9 (Cell Signaling; rat specific, rabbit polyclonal) were used, with GAPDH (anti-GAPDH antibody from Research Diagnostics, Concord, MA) as an internal control.

Caspase-9 and caspase-3 activity assay

The procedures were the same as previously described in detail (Han et al., 2001; Han et al., 2004a; Han et al., 2004b; Wang et al., 2002).

MTT assay for cell viability

Cell Proliferation Kit I [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT); Roche Molecular Biochemicals, Laval, PQ, Canada] was used to quantify survival of cells from oxidative stress (Han et al., 2001; Han et al., 2004a; Han et al., 2004b; Wang et al., 2002).

Enzyme-linked immunosorbent assay (ELISA)

The Cell Death Detection ELISA kit (Ro e Molecula Biochemicals) was employed to quantify DNA fragmentati on the sis of anti dy detection of free histone and fragmented DNA (Han e l., 2001; Ha t al., 20 a; Han et al., 2004b; Wang et al., 2002).

Terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)

DNA fragmentati of individual cells detected in situ by TUNEL with the In Situ Cell Death Detection it, Fluorescein (Roche Molecular Biochemicals) (Han et al., 2001; Han et al., 2003; n et al., 2004a; Han et al., 2004b; Wang et al., 2002).

Data analysis

Grou data are exp ssed as mean ± s.e.m. Statistical comparisons (performed using ANO A followed Dunnett's method) were carried out using Microsoft Excel. A two-tai $P<0.05$ was taken to indicate a statistically significant difference.

e authors thank XiaoFan Yang for excellent technical support. This work was supported in part by the Natural Sciences and Engineering Research Council of Canada and Fonds de la Recherche de l'Institut de Cardiologie de Montreal, awarded to Z. Wang, and by the National Nature Science Foundation of China (30430780), the Foundation of National Department of Science and Technology of China (2004CCA06700), and National Basic Research Program of China (973 Program; 2007CB512000/2007CB512006) awarded to B. Yang. Z. Wang is a senior research scholar of the Fonds de Recherche en Sante de Quebec.

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