MicroRNA-1 Regulates Cardiomyocyte Apoptosis by Targeting Bcl-2

Yehua Tang,1 MD, Jiaoyang Zheng,2 MD, Yan Sun,3 MD, Zonggui Wu,1 MD, Zhimin Liu,2 MD, and Gaozhong Huang,1,4 MD

SUMMARY

MicroRNA-1 (miR-1) is preferentially expressed in cardiac muscles, and the expression has been demonstrated to be involved in cardiac development and cardiovascular diseases. Here we report that miR-1 is closely related with ischemia/reperfusion injury in a rat model. The level of miR-1 is inversely correlated with Bcl-2 protein expression in cardiomyocytes of the I/R rat model. In vitro, the level of miR-1 was dramatically increased in response to H2O2. Overexpression of miR-1 facilitated H2O2-induced apoptosis in cardiomyocytes. Inhibition of miR-1 by antisense inhibitory oligonucleotides caused marked resistance to H2O2. Through bioinformatics, we identified the potential target sites for miR-1 on the 3’ UTR of Bcl-2. miR-1 significantly reduced the expression of Bcl-2 in the levels of mRNA and protein. The post-transcriptional repression of Bcl-2 was further confirmed by luciferase reporter experiments. These data demonstrated that miR-1 plays an important role in the regulation of cardiomyocyte apoptosis, which is involved in post-transcriptional repression of Bcl-2. (Int Heart J 2009; 50: 377-387)

Key words: miR-1, Bcl-2, Apoptosis, Cardiomyocyte

MICRORNAs (miRNAs or miRs) are recently discovered small, endogenous, single-stranded noncoding RNAs of 18–25 nucleotides.1,2) They post-transcriptionally regulate gene expression by hybridization to messenger RNA (mRNA), leading to translational repression or degradation of the target mRNA. miRNAs have emerged as one of the central players of gene expression regulation.2,3) While the role of miRNAs in oncogenesis and cardiac development has been well appreciated over the past few years, the implications of miRNAs in the pathological processes of the cardiovascular system have only been recognized very recently.4) The research on miRNAs in relation to cardiovascular disease

From the Departments of 1 Cardiology, 2 Endocrinology, Shanghai Changzheng Hospital, Second Military Medical University, 3 Department of Gastroenterology, Ruijin Hospital, School of Medicine, Shanghai Jiao Tong University, 4 Department of Gerontology, Sixth People’s Hospital, Shanghai Jiaotong University, Shanghai, China.

* Authors that contributed equally to this study.

Address for correspondence: Department of Gerontology, Sixth People’s Hospital, Shanghai Jiaotong University, No. 600, Yisan Road, Shanghai, 200233, and Department of Cardiology, Shanghai Changzheng Hospital, Second Military Medical University, No. 415, Fengyang Road, Shanghai, 200003, China.

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has become a rapidly evolving field. Many researchers have identified some microRNAs that are involved in cardiac disease.\textsuperscript{5}) Reports about microRNA targeted genes are very limited. Here, we attempted to elucidate the mechanism of microRNA-regulating apoptosis in cardiomyocytes, with particular emphasis on identifying apoptosis associated genes targeted by miRNA in cardiomyocytes.

According to previous reports, the expression profile of microRNAs appears to be tissue-specific or developmental stage-specific expression patterns, and their sequences exhibit evolutionary conservation.\textsuperscript{1}) Both have been found in most animal species, from Drosophila to human, indicating that they are evolutionally conserved. Among the known miRNAs, miR-1 is believed to be specifically expressed in adult cardiac and skeletal muscle tissues. miR-1 can regulate myogenesis by controlling distinct aspects of the differentiation process.\textsuperscript{2,4}) MiR-1 promotes myogenic differentiation. With the exception of regulating cardiac development, miR-1 had been proposed to be involved in regulating cardiomyocyte apoptosis.\textsuperscript{3,4}) The MiR-1 level was significantly increased in response to oxidative stress and this increase reduced the protein levels of two anti-apoptotic molecules, heat shock protein-60 (HSP60) and HSP70, without changing their transcript levels. It has been predicted that each single miRNA can have > 1,000 target genes and each single protein-coding gene can be regulated by multiple miRNAs. Also, there are many genes involved in cardiomyocytes, such as the Bcl-2 family. The relationship between the Bcl-2 family and miR-1 is not fully understood in cardiomyocytes. Apoptosis is believed to play a crucial role in many cardiovascular diseases, and is an important means with which to maintain cardiomyocyte homeostasis. In the present study, we have attempted to elucidate the mechanism of cardiomyocyte apoptosis with respect to miR-1 and the target gene.

**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.

**Myocardial ischemia/reperfusion rat model:** Male mice were subjected to myocardial ischemia/reperfusion (I/R) as previously described.\textsuperscript{3}) Following a 30-minute coronary artery occlusion, the suture was removed to allow coronary reperfusion followed by closure of the chest wall. After 24 hours of coronary artery reperfusion, the heart was excised and perfused as previously described.\textsuperscript{2})

**Synthesis of miRNAs and sequences of miRNA inhibitors:** MiR-1 and its mutant
constructs were synthesized by Applied Biosystems. The sequences of miR-1 and its inhibitors (AMOs; anti-miRNA oligonucleotides) used in our studies are the exact antisense copies of their respective mature miRNA sequences: 3’-UAU-GUGUGAAGAAUAGUAAGGU-5’ for rat miR-1. All the nucleotides in the AMO-1 for miR-1 contain 2’-O-methyl modifications at every base and a 3’ C3-containing amino linker.

**Construction of 3’ UTR luciferase reporter vectors:** To generate reporter vectors bearing miRNA-binding sites, we generated direct match miR-1 sites and the sequences around the putative target sites for these miRNAs in the 3’UTRs of Bcl-2. These insert sequences were cloned into the multiple cloning sites in the pMIR-REPORT™ luciferase miRNA expression reporter vector (Ambion, Inc.). Nucleotide-substitution mutations (MT) were carried out using direct oligomer synthesis for miR-1, and PCR-based methods for the 3’ UTRs of Bcl-2. The substitution nucleotides were so designed to avoid producing new binding sites for other miRNAs potentially existing in HEK293 cells. All constructs were sequencing verified.

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

**Quantitative real-time RT-PCR assay:** Myocardial samples were homogenized in RNA lysis buffer. RNA was extracted using a MagMAX™-96 Total RNA Isolation Kit (Ambion, USA). According to the manufacturers’ instruction, 5 mg samples are homogenized in 100 μL of prepared Lysis/Binding Solution. The mRNA is captured by magnetically RNA binding beads and then the total RNA is treated by TURBO DNase Solution and clean-up by Wash Solution. The quality of RNA is valued by Real-time PCR. The real-time PCR amplification was carried out using QuantiTect SYBR green (Qiagen, CA, USA), according to the manufacturer’s instructions. The CT (threshold cycle) value of caspase-12 amplification was normalized to GAPDH control.

**Statistical analysis:** All experiments, except immunoblots, were performed in triplicate, and the results are expressed as the mean ± SD. The comparative CT method was applied in the quantitative real-time RT-PCR assay, according to the delta-delta CT method. The data were analyzed with Student’s t-test or by one-way analysis of variance (ANOVA), and the criterion for statistical significance was P < 0.05.

### Results

**The level of miR-1 was inversely correlated with Bcl-2 protein expression in I/R rat model cardiomyocytes:** To determine which miRNA was closely related with
I/R injury, an I/R rat model was constructed. Total RNA from cardiomyocytes of the I/R rat model was used for hybridization on each miRNACHIP microarray chip containing 368 probes in triplicate, corresponding to 245 human and rat

Figure 1. Correlation between miR-1 and Bcl-2.
(A) I/R rat model cardiomyocytes were analyzed by miRNA CHIP microarray chip. The cardiac specific miRNAs analyzed included miR-1, miR-21, miR-126, miR-133, miR-195, and miR-208.
(B) The coefficient of correlation was nearly 94% between Bcl-2 and miR-1. Bcl-2 expression is on the abscissa while the miR-1 level by miRNA chip is on the ordinate.
miRNA genes. Raw data were normalized and analyzed using GENESPRING software (Silicon Genetics, CA). As shown in Figure 1A, the level of miR-1 was increased by nearly 6-fold, when compared with the sham operation group. Next, we evaluated the correlation between the expression levels of miR-1 and the Bcl-2 protein levels in I/R rat cardiomyocytes. Using miRNA-CHIP and

**Figure 2.** Cell viability and apoptosis under various treatments. 
(A) MiR-1 expression levels were analyzed by real-time RT-PCR, while cells were transfected with WT miR-1 or MT miR-1. (B) The concentration-dependent effect of H$_2$O$_2$ on cardiomyocytes. Cell viability was analyzed by MTS assay under different H$_2$O$_2$ concentrations. (C) While cells were treated with miRNA or/and AMO-1, the amount of apoptosis was analyzed by Annexin V/PI binding assay. The data represent 3 independent experiments. Fold increase was determined by calculation with respect to control (untreated cells). Significant differences are indicated by asterisks: *P < 0.05 and **P < 0.01, compared with untreated control cells.
Western blotting, we analyzed a set of 15 samples, composed of 12 I/R injury samples and 3 sham operation samples. In sham cardiomyocytes the levels of miR-1 were low, while Bcl-2 protein was expressed at high levels. Moreover, in all I/R samples we found a highly concordant inverse correlation between the miR-1 and Bcl-2 levels, $R^2 = 0.9369$ (Figure 1B).

**MiR-1 regulates apoptosis induced by H\textsubscript{2}O\textsubscript{2} in cardiomyocytes:** Rat cardiomyocytes were divided into the following 3 groups: control (untransfected cells), wild-type (WT) miR-1, and mutant (MT) miR-1 groups, and they were subjected to transfection procedures. The level of miR-1 was analyzed 48 hours after transfection. All measurements were performed at this time in order to exclude any influences from transfection time. The primer of miR-1 for real-time RT-PCR was designed to be complementary to WT miR-1, which was not designed for detecting the level of MT miR-1 (Figure 2A). MiR-1 transfection resulted in an approximately 3- to 4-fold increase in miR-1 levels. Incubation of cells with H\textsubscript{2}O\textsubscript{2} (200 $\mu$M) caused an approximately 2- to 3-fold increase of miR-1 (Figure 2A). The expression level of miR-1 in cells treated with H\textsubscript{2}O\textsubscript{2} and WT miR-1 was significantly higher than that in cells treated with miR-1 or H\textsubscript{2}O\textsubscript{2} alone (Figure 2A).

Cardiomyocytes were exposed to increasing concentrations of H\textsubscript{2}O\textsubscript{2} (0.1-1 mM) and cardiomyocyte viability was assessed by MTS. The loss of cell viability was accompanied by an increase in H\textsubscript{2}O\textsubscript{2} concentration. Also, overexpression of WT miR-1 dramatically decreased cell viability (Figure 2B). Next, we attempted to determine whether the cell death was caused by apoptosis. The rate of apoptosis was analyzed by Annexin V/PI binding assay (Figure 2C). To present the results as clearly as possible, the apoptosis rate is shown as cells treated with 0.4 mM H\textsubscript{2}O\textsubscript{2}. Overexpression of miR-1 induced apparent apoptotic cell death. Also, the apoptotic-inducing effect was not observed in cells transfected with MT miR-1. Interestingly, cotransfection of WT miR-1 and MT miR-1 largely prevented the apoptosis induced by WT miR-1 alone. If cells cotransfected with mutant Bcl-2 and WT miR-1, apoptosis induced by miR-1 was attenuated compared with cells transfected with WT miR-1 alone (refer to supplemental data). To further verify the pro-apoptotic effects of miR-1, cardiomyocytes were transfected with 2’-O-methyl antisense inhibitory oligonucleotides (AMOs) against MT miR-1 (AMO-1). AMO-1 would compromise the function of miR-1. AMO-1 reduced H\textsubscript{2}O\textsubscript{2}-induced apoptosis, whereas miR-1 facilitated it (Figure 2C). Cotransfection of WT miR-1 and AMO-1 failed to alter H\textsubscript{2}O\textsubscript{2}-induced apoptosis, which further demonstrated miR-1 had a pro-apoptotic effect on cardiomyocytes.

**Identification of potential target sites interacting with miR-1:** Here we have demonstrated that miR-1 had a pro-apoptotic effect on cardiomyocytes, which was
possible due to regulation of apoptotic or survival factors by binding the 3’ UTR of the target gene. To test this assumption, we used a computational and bioinformatics-based approach to predict the putative target related to apoptosis. According to Targetscan algorithms, we identified important candidate targets for miR-1: Bcl-2. There was a target site for miR-1 at 2439-2463 of Bcl-2 3’ UTR. The specific binding site and parameters were:

UAACUCACUUGGGAAACAUUUUCCCC   Bcl-2 3’ UTR
UAUGUGUGAAGAAUGUA - -GGU   miR-1

Figure 3. Expression of Bcl-2 at the level of mRNA and protein. 
(A) The expression of Bcl-2 mRNA was analyzed by real-time RT-PCR. The data represent 3 independent experiments. Fold increase of Bcl-2 was determined by calculation with respect to control (untreated cells). Significant differences are indicated by asterisks: *P < 0.05 and **P < 0.01, compared with cells transfected with control. (B) The expression of Bcl-2 at the level of protein was assayed by Western blot analysis.
MiR-1 regulates Bcl-2 expression in both mRNA and protein levels: To verify Bcl-2 is the target of miR-1, we first determined the effects of miR-1 on Bcl-2 mRNA levels by real-time RT-PCR. As shown in Figure 3A, WT miR-1 significantly reduced the levels of Bcl-2 mRNA in cardiomyocytes in the absence and presence of \( \text{H}_2\text{O}_2 \). In contrast, MT miR-1 did not affect the levels of Bcl-2 mRNA. Cotransfection of WT miR-1 and MT miR-1 attenuated the effect. In the

**Figure 4.** Activity of 3′ UTR luciferase reporter plasmid. Relative activity was determined by calculation with respect to control (untreated cells). Significant differences are indicated by asterisks: *\( p < 0.05 \) and **\( p < 0.01 \), compared with cells transfected with control.
presence of AMO-1, the repression effect of miR-1 on Bcl-2 was compromised. Next, we further assayed the effect of miR-1 on Bcl-2 protein levels by Western blotting. Overexpression of miR-1 consistently and markedly decreased the expression of Bcl-2 protein (Figure 3B). Coapplication of miR-1 and AMO-1 attenuated this effect. While cells were cotransfected with WT miR-1 and MT miR-1, the expression of Bcl-2 protein was not significantly decreased. Also, application of AMO-1 alone increased the levels of Bcl-2 at both the protein and mRNA levels, which further demonstrated the repression effect of miR-1 on Bcl-2.

**Verification of interaction between miR-1 and Bcl-2:** A luciferase reporter plasmid was used to verify the putative binding sites for miR-1 in 3’ UTR of Bcl-2. Wild-type 3’ UTR of Bcl-2 were constructed into the 3’ UTR of a luciferase reporter plasmid. A mutant target sequence of Bcl-2 was also fused to the reporter plasmid. These constructed chimeric vectors were cotransfected with miR-1 into HEK 293 cells. The presence of an interaction miRNA::mRNA would reduce the firefly luciferase activity. Because miR-1 is known to be muscle-specific, HEK293 expresses these microRNAs very minimally. HEK 293 cells were used to verify the interaction between miR-1 and Bcl-2, which should exclude effects from endogenous miR-1 and mimic a real interaction between them. As shown in Figure 4, data on luciferase reporter activities show the interaction between miR-1 and Bcl-2 3’UTRs. In contrast to WT Bcl-2, the mutated target sequences of Bcl-2 fused to the 3’UTR of luciferase were not responsive to miR-1. MT miR-1 was designed to contain complementary sequences to MT 3’UTRs of Bcl-2. As shown in Figure 4, MT miR-1 had no effect on the WT target 3’UTR sequences of Bcl-2, but could efficiently repress the luciferase activities of MT Bcl-2.

**DISCUSSION**

The search for the basic mechanisms responsible for the development and progression of heart disease has been exhaustive. Apoptosis causes loss of contractile cells, compensatory hypertrophy of myocardial cells, and reparative fibrosis. Because a reduction of contractile material is a prominent feature in heart failure, modification of apoptosis in the myocardium might provide a new therapeutic target for cardiovascular diseases. Bcl-2 is the founding member of a growing family whose members have emerged as important regulators of cardiomyocyte apoptosis. Among Bcl-2 family members, Bcl-2 is a central player in the genetic program of cardiomyocytes favoring survival by inhibiting cell death. There are many apoptosis regulating mechanisms, which are associated with Bcl-2 in cardiomyocytes. In the present study, we have attempted to iden-
tify the regulation mechanism of Bcl-2 in the field of microRNA.

MicroRNAs are an evolutionally conserved class of small regulatory RNAs that have gained status recently as important regulators in cardiac developmental and pathological processes. The global role of miRNA function in the heart has been addressed by conditionally inhibiting miRNA maturation in the murine heart and has revealed that miRNAs have an essential role during heart development.\(^1\) MiRNA expression-profiling studies have demonstrated that the expression levels of specific miRNAs change in diseased human hearts, pointing to their involvement in cardiomyopathies.\(^2\) Furthermore, studies on specific miRNAs in animal models have identified distinct roles for miRNAs both during heart development and under pathological conditions, including the regulation of key factors important for cardiogenesis, the hypertrophic growth response, and cardiac conductance. Cumulatively, these findings indicate clearly that miRNAs are important regulators of gene expression in heart development, function, and pathology. Among the known miRNAs, miR-1 is believed to be specifically expressed in adult cardiac and skeletal muscle tissues. One important characteristic of miR-1 is regulating apoptosis of cardiomyocytes. However, previous research merely speculates so further studies are needed for verification. Moreover, it should also be noted that previous studies merely provided indirect evidence for the interactions between miR-1 and target genes, and therefore, more rigorous experimentation is required to fully establish the relationships.

In the present research, we demonstrated that miR-1 regulated apoptosis in cardiomyocytes. While cardiomyocytes were exposed to oxidative stress, the apoptosis rate was decreased in miR-1 overexpression cells. According to a bioinformatics analysis, we identified a potential target site for miR-1 in the 5' UTR of Bcl-2. Also, our data demonstrated that Bcl-2 was silenced by miR-1 at the protein and mRNA levels. Earlier, miRNAs were thought to primarily repress their targets at the protein level without affecting mRNA stability. Increasing evidence indicates that miRNAs silence genes by multiple mechanisms including degrading their target mRNAs. Our observations seem to be in line with multiple mechanisms of action. However, it is presently unclear what determines the exact mechanisms of miRNA actions. Thus, we verified the interaction between Bcl-2 and miR-1 through 3' UTR luciferase reporter plasmid.

Collectively, our study revealed a novel aspect of the cellular functions of muscle-specific miR-1 regulation of apoptosis and survival in cardiomyocytes. Post-transcriptional repression of Bcl-2 by miR-1 is probably one of the mechanisms underlying their regulation of apoptosis versus survival. Our present and previous studies revealed pathological elevations of miR-1 levels in cardiomyocytes in conditions favoring apoptosis (ischemia and oxidative stress). These results could help us better understand cardiac pathology and promote more ef-
References