MicroRNA-1 aggravates cardiac oxidative stress by post-transcriptional modification of the antioxidant network

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Abstract Oxidative stress plays an important role in cardiovascular diseases. Studies have shown that miR-1 plays an important role in the regulation of cardiomyocyte apoptosis, which can be the result of oxidative stress. This study was designed to determine whether increased miR-1 levels lead to alterations in the expression of proteins related to oxidative stress, which could contribute to heart dysfunction. We compared cardiac function in wild-type (WT) and miR-1 transgene (miR-1/Tg) C57BL/6 mice ($n \geq 10$ /group). Echocardiography showed that stroke volume (SV), ejection fraction (EF), and fractional shortening (FS) were significantly decreased in miR-1/Tg mice. Concomitantly, the level of reactive oxygen species (ROS) was elevated in the cardiomyocytes from the miR-1/Tg mice, and activities of lactate dehydrogenase (LDH) and creatinine kinase (CK) in plasma were also increased in the miR-1/Tg mice. All of these changes could be reversed by LNA-anti-miR-1. In the cardiomyocytes of neonatal Wistar rats, overexpression of miR-1 exhibits higher ROS levels and lower resistance to H_2O_2 -induced oxidative stress. We demonstrated that SOD1, Gclc, and G6PD are novel targets of miR-1 for post-transcriptional repression.

MicroRNA-1 post-transcriptionally represses the expression of SOD1, Gclc, and G6PD, which is likely to contribute to the increased ROS level and the susceptibility to oxidative stress of the hearts of miR-1 transgenic mice.

Keywords MicroRNAs · Oxidative stress · Post-transcriptional modification

Introduction

According to the World Health Organization (WHO), cardiovascular disease is the leading cause of death worldwide, accounting for approximately 30 % of all deaths. In particular, ischemic heart disease is the leading cause of global mortality (Maejima et al. 2011; Touyz and Briones 2011; Cheng et al. 2011; Trachtenberg and Hare 2009). Oxidative stress plays an important role in cardiovascular diseases, such as hypertension, cardiac hypertrophy, atherosclerosis, and heart failure (Maejima et al. 2011; Touyz and Briones 2011; Cheng et al. 2011; Trachtenberg and Hare 2009). Oxidative stress can damage proteins, lipids, and DNA. Cells are equipped with antioxidant machinery, such as the superoxide dismutase (SOD)-catalase system and the glutathione and thioredoxin systems. Under physiological conditions, the balance between oxidative stress and antioxidant activities is well maintained. Only when the oxidative stress levels exceed the antioxidant defense capacity does oxidative damage result, whereas the low levels of oxidative stress may stimulate the defense network, thereby protecting cells against the forthcoming oxidative stress.

MicroRNAs consist of ~22 endogenous nucleotides that regulate gene expression by annealing to target messenger RNAs, inhibiting translation or promoting messenger RNA (mRNA) degradation (Denli et al. 2004). Among the ~2000

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microRNAs identified in humans, several, including miR-1, are muscle-specific. Deep sequencing of miRNAs from heart tissue has revealed that the abundance of miR-1 accounts for 40 % of all miRNAs. MiR-1 is involved in cardiac development and apoptosis and is elevated in the hearts of individuals with coronary artery diseases, ischemic arrhythmias, and heart failure (Ai et al. 2010; Shan et al. 2009; Lu et al. 2009; Anderson and Mohler 2007; Yang et al. 2007). Several studies have shown that miR-1 plays an important role in the regulation of cardiomyocyte apoptosis through post-transcriptional repression of signal proteins, such as Bcl-2 and IGF-1 (Tang et al. 2009; Yu et al. 2008). Apoptosis is the result of oxidative stress. Coronary artery disease, ischemic arrhythmias, and heart failure are all oxidative stress-related diseases. One of the questions we asked is whether miR-1 has any relationship with the production of oxidative stress. Database screening indicated that miR-1 has potential complementary sites in the 3' UTRs of anti-oxidant genes Gclc, SOD, and G6PD. To gain insight into potential molecular changes that may occur in the heart due to overexpression of miR-1, we developed a miR-1 transgenic (miR-1/Tg) mouse model carrying the cardiac-specific α myosin heavy chain (α -MHC) promoter in mice. This study was designed to determine whether increased miR-1 levels lead to alterations in the expression of proteins related to oxidative stress, which could contribute to heart dysfunction.

Methods

Animals

Three-month-old male wild-type (WT) and cardiac-specific miR-1 transgenic (Tg) mice in a mixed C57BL/6 background were used for all of the experiments. MiR-1 transgenic mice were generated as previously reported in our laboratory (Tang et al. 2009; Yu et al. 2008). The mice were housed in groups in a temperature-controlled room with a 12/12 light/dark cycle. Food and water were provided ad libitum. The animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the China National Institutes of Health. All animal procedures were approved by the Institutional Animal Care and Use Committee at Harbin Medical University (no. HMUIRB-2008-06) and the Institute of Laboratory Animal Science of China (A5655-01). All procedures conform to the Directive 2010/63/EU of the European Parliament.

Ischemia/reperfusion (I/R) model

C57BL/6 mice were anesthetized with pentobarbital sodium (50 mg/kg ip). The animal was orally intubated with a 20-gauge tube and ventilated (mouse ventilator, UGO BASILE,

Biological Research Apparatus, Italy) at a respiratory rate of 100 breaths/min with a tidal volume of 0.3 ml. The left anterior descending (LAD) artery was occluded at a position 2 mm from the tip of the left auricle. Myocardial ischemia was verified by blanching of the LV and by changes in the electrocardiogram. Following 30 min of LAD occlusion and 2 h of reperfusion, the hearts were removed for further study.

Neonatal rat cardiomyocyte culture

Primary cultures of neonatal rat cardiomyocytes were prepared as previously described (Ai et al. 2010; Shan et al. 2009; Lu et al. 2009; Anderson and Mohler 2007; Yang et al. 2007). Briefly, hearts from Wistar rats (1–3 days old) were minced and dissociated with 0.25 % trypsin. Dispersed cells were seeded at 2×10^5 cells/cm² in 60-mm culture dishes with Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and then cultured in a 5 % CO₂ incubator at 37 °C.

Transthoracic echocardiography

Echocardiography was carried out using an ultrasound machine (Vivid 7 GE Medical) equipped with a 10-MHz phased-array transducer to test the cardiac function of mice.

Synthesis and administration of locked nucleic acid anti-sense miR-1

The anti-sense sequence of miR-1 (LNA-antimiR-1) was provided by Exiqon (Vedbaek, Denmark), and five nucleotides or deoxynucleotides at both ends of the anti-sense molecules were locked (LNA; the ribose ring is constrained by a methylene bridge between the 2'-O- and the 4'-C atoms). The sequence of LNA antimiR-1 is 5'-ACTTCTTTACATTCC-3'. The LNA-control was synthesized with the following sequence: 5'-ACGTCTATACGCCCA-3'. LNA-antimiR-1 or LNA-control sequence at a dose of 1 mg/kg was intravenously injected via the tail vein into the mice two times a week for 4 weeks before analysis. The animals ($n=6$ in each group) were killed by cervical vertebrae dividing and the hearts were achieved.

Quantitative real-time RT-PCR analysis

Total RNA samples from cultured cardiomyocytes and hearts were prepared using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Samples of total RNA (2 μ g) were reverse-transcribed using the RNA PCR Kit (Takara Biotechnology, Japan), and the resulting cDNA was used as a PCR template. The mRNA levels were determined by real-time PCR with the ABI PRISM 7700 Sequence Detector (Applied Biosystems), according to the manufacturer's

instructions. Predesigned gene-specific primer and probe sets (TaqMan Gene Expression Assays) were used. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA was amplified as an internal control. The relative gene expression level (the amount of target, normalized to the endogenous control gene) was calculated using the comparative Ct method formula: $2^{-\Delta\Delta C_t}$.

Western blot analysis

The protein samples were extracted from the hearts of mice or the cultured rat cardiomyocytes for immunoblotting analysis using essentially the same procedures as described in detail elsewhere (Pan et al. 2012). The protein content was determined via the BCA Protein Assay Kit using bovine serum albumin as the standard. The protein sample (~50 µg) was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10 % polyacrylamide gels) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA). The sample was incubated overnight at 4 °C with the primary antibodies at a 1:1000 dilution. Affinity-purified rabbit polyclonal anti-Gclc (Abcam), rabbit polyclonal anti-SOD (Abcam), and rabbit polyclonal antibodies to G6PD (Santa Cruz Biotechnology Inc.) were used as the primary antibodies. The next day, the membrane was incubated with secondary antibodies (Molecular Probes) diluted in phosphate-buffered saline (PBS) for 1 h at room temperature. Finally, the membrane was rinsed with PBS before scanning using the Infrared Imaging System (LI-COR Biosciences). GAPDH was used as an internal control for equal input of protein samples using the anti-GAPDH antibody. Western blot bands were quantified using QuantityOne software by measuring the band intensity (area×OD) for each group and were normalized to GAPDH. The final results are expressed as fold changes by normalizing the data to the control values.

Cell viability

Cell viability was assessed using the Invitrogen Viability Assay Kit, which is based on the simultaneous determination of live and dead cells with the calcein AM and ethidium homodimer-1 probes, which are specific for intracellular esterase activity and membrane integrity, respectively. Fluorescence imaging of the cells (live cells were labeled green, whereas the nuclei of dead cells were labeled red) was performed with a fluorescence microscope (BZ-9000; Keyence).

Detection of ROS by flow cytometry

The reactive oxygen species (ROS) levels of cardiomyocytes were quantified using the ROS assay kit (Beyotime Institute of Biotechnology, China). Cells were harvested by trypsin and exposed for 20 min at 37 °C to 10 µM DCFH-DA, which

became fluorescent when oxidized to DCF within the cells. Labeled cells were washed twice in PBS and then subjected to cytometry analysis (emission, 488 nm; excitation, 525 nm). Flow cytometry was performed using FACSaria (BD), and the ROS level was represented by fluorescence intensity. A total of 10,000 cells were analyzed in each individual experiment.

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

The 3'-UTR regions of the SOD1, Gclc, and G6PD containing the predicted binding sites for miR-1, wild or mutant (SOD1 1–7 ACATTCC mutated to TGTAAGG, Gclc 326–332 CATT CCA mutated to GTAAGGT, and G6PD 527–533 ACATTCC mutated to TGTAAGG), were cloned into the multiple cloning sites in the pMIR-REPORTTM luciferase miRNA expression reporter vector (Ambion, Inc.). The sense and anti-sense strands of the oligonucleotides were annealed by adding 2 µg of each oligonucleotide to 46 µl of annealing solution (100 mM potassium acetate, 30 mM Hepes-KOH, pH 7.4, and 2 mM magnesium acetate), followed by incubation at 90 °C for 5 min and then at 37 °C for 1 h. The annealed oligonucleotides were digested with HindIII and SpeI and ligated into HindIII and SpeI sites.

Transfection of miRNAs and the luciferase assay

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

For the 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 h after transfection with a dual luciferase reporter assay kit (Promega) on a luminometer (Lumat LB9507).

Statistical analysis

All the data are expressed as means ± SD. Statistical analysis was performed using two-way analysis of variance (ANOVA), followed by Student's *t* test. Differences were considered to be significant when $p < 0.05$.

Results

Cardiac function decreased in miR-1/Tg mice

Echocardiography examination was performed first to assess cardiac function. The results showed that ejection fraction

(EF), fractional shortening (FS), and stroke volume (SV) were significantly decreased in miR-1/Tg mice compared with WT mice, indicating impaired cardiac function (Table 1). Meanwhile, the left ventricular diastolic diameter (LVDd), interventricular septum systolic thickness (IVSs), and end diastolic volume (EDV) tended to be decreased, whereas the left ventricular systolic diameter (LVSD), interventricular septum diastolic thickness (IVSd), and end systolic volume (ESV) tended to be increased ($*P<0.05$ vs. WT); however, the difference in IVSs and EDV between the two groups was not statistically significant (Table 1). Histological analysis of the heart in miR-1 Tg mice compared with WT mice was particularly demonstrated in a previous report by our laboratory (Pan et al. 2012). These findings indicated that systolic and diastolic dysfunction and heart failure development were present in miR-1/Tg mice.

LNA-anti-miR-1 reverses the cardiac damage in miR-1/Tg mice

The expression level of miR-1 was markedly elevated (~4.1-fold) in RNA samples from heterozygous litters of miR-1/Tg mice compared with those from WT mice. Additionally, the application of LNA-anti-miR-1 to miR-1/Tg mice significantly decreased miR-1 levels (Fig. 1a, $^{#}P<0.05$ vs. miR-1). To investigate whether cardiac injury existed in miR-1/Tg mice, we examined the ROS level in cardiomyocytes and the plasma lactate dehydrogenase (LDH) and CK activities. These markers all indicated myocardial damage. The ROS level was elevated 1.36-fold in cardiomyocytes from miR-1/Tg mice compared with those from WT mice (Fig. 1b, $*P<0.05$ vs. WT). Blood plasma LDH and CK activities were increased

by 1.6- and 1.55-fold, respectively, in miR-1/Tg mice (Fig. 1c, d). All these changes could be reversed by LNA-anti-miR-1 ($^{#}P<0.05$ vs. miR-1), while LNA-con did not display the effect suggesting the specific inhibition of miR-1 induced by LNA-anti-miR-1.

Forced overexpression of miR-1 in cardiomyocytes yields higher ROS levels and lower resistance to oxidative stress

We further examined the ROS levels in neonatal rat cardiomyocytes with or without transfection of miR-1 by flow cytometry. The fluorescence intensity increased from 3093.0 to 7266.0 in cardiomyocytes overexpressed with miR-1. This result indicated that the ROS level was increased 2.3-fold in miR-1-transfected cells, which could be inhibited by its anti-sense miRNA oligonucleotide (AMO)-1 (Fig. 2a and b, $*P<0.05$ vs. NC; $^{#}P<0.05$ vs. miR-1). Cell viability in different groups was also monitored. Little change in cell viability was observed among negative control (NC), miR-con, miR-1, miR-1+AMO-1, and miR-1+AMO-con groups (Fig. 2c, e). miR-con was a non-specific miRNA used as negative control. To investigate the resistance to oxidative stress, cardiomyocytes were treated with 100 μ M H_2O_2 for 12 h. Cell viability in the H_2O_2 -treated group was reduced to 70.58 % compared with NC group. Overexpression of miR-1 increased this reduction to 35.67 %, which was inhibited by co-transfection of AMO-1 (Fig. 2d, f; $P<0.05$). From these results, we deduced that the resistance to external oxidative stress was significantly decreased in cardiomyocytes with miR-1 overexpression.

SOD1, Gclc, and G6PD are novel targets of miR-1 for post-transcriptional repression

Elevated ROS levels and cardiac injury accompanied by decreased cardiac function suggested that miR-1 may affect the redox system in the body. To test this hypothesis, we identified three relevant targets for miR-1 among the known important redox enzyme genes, namely SOD1, Gclc, and G6PD. The 3'-untranslated regions of SOD1, Gclc, and G6PD all contain seven nucleotides that are complementary to the first eight nucleotides from the 5' end of miR-1 (Fig. 3, upper panel).

Previous studies have indicated that miRNA-binding sites are transferable and sufficient to confer miRNA-dependent gene silencing. We inserted the wild and mutant 3'-UTRs of SOD1, Gclc, and G6PD into the 3'-UTR of a luciferase reporter plasmid containing a constitutively active promoter to determine the effects of miR-1 on reporter expression. Wild-type 3'-UTR of SOD1, Gclc, and G6PD cotransfection of miR-1 with the plasmid (Fig. 3, lower panel) into HEK293 cells consistently produced less luciferase activity than transfection of the plasmid alone or cotransfection with miR-con ($*P<0.05$), whereas the mutant 3'-UTR did not reduce

Table 1 Echocardiography of wild-type control and miR-1 transgenic mice

Group	WT (n=10)	miR-1/Tg (n=10)
EF (%)	78.18 $\pm$ 9.23	46.39 $\pm$ 6.21*
FS (%)	40.46 $\pm$ 6.82	19.63 $\pm$ 2.86*
SV (ml)	0.09 $\pm$ 0.02	0.04 $\pm$ 0.01*
IVSd (mm)	1.06 $\pm$ 0.19	1.17 $\pm$ 0.13*
IVSs (mm)	1.27 $\pm$ 0.22	1.2 $\pm$ 0.24
LVDd (mm)	3.42 $\pm$ 0.33	3.34 $\pm$ 0.53*
LVSD (mm)	2.03 $\pm$ 0.27	2.77 $\pm$ 0.43*
EDV (ml)	0.11 $\pm$ 0.03	0.09 $\pm$ 0.02
ESV (ml)	0.03 $\pm$ 0.01	0.06 $\pm$ 0.01*

n=10 mice in each group. Data are expressed as mean $\pm$ SD

EF ejection fraction, FS fractional shortening, SV stroke volume, IVSd interventricular septum diastolic thickness, IVSs interventricular septum systolic thickness, LVDd left ventricular diastolic diameter, LVSD left ventricular systolic diameter, EDV end diastolic volume, ESV end systolic volume

* $p<0.05$

Fig. 1 MiR-1 LNA reversed the degree of cardiac damage in miR-1 mice. MiR-1 LNA reduced ROS levels and the degree of cardiac damage in miR-1 mice. **a** MiR-1 levels were significantly increased in the hearts of miR-1 transgenic mice compared with those of normal controls, and miR-1 LNA decreased miR-1 levels in the hearts of miR-1 transgenic mice, while LNA-con had no effect on miR-1 levels. **b** ELISA results showed that the ROS level was increased in the hearts of miR-1 transgenic mice compared with the normal control, and miR-1 LNA decreased ROS levels in the hearts of miR-1 transgenic mice, while LNA-con did

not decrease ROS levels. **c** LDH activity was increased in the hearts of miR-1 transgenic mice compared with those of the normal controls, and miR-1 LNA decreased LDH activity in the hearts of miR-1 transgenic mice, while LNA-con had no effect on LDH activity. **d** CK activity was increased in the hearts of miR-1 transgenic mice compared with those of the normal controls, and miR-1 LNA decreased CK activity in the hearts of miR-1 transgenic mice, while LNA-con did not decrease CK activity. Mean $\pm$ SEM. $n=6$ mice in each group. * $P<0.05$ versus WT; # $P<0.05$ versus WT miR-1; unpaired Student's t test

luciferase activity. Transfection of AMO-1 into the cells eliminated the silencing effects of miR-1 on the activities of the wild-type SOD1-, Gclc-, or G6PD-luciferase chimeric vector and target sequences (# $P<0.05$ vs. miR-1).

Overexpression of miR-1 decreased Gclc, SOD1, and G6PD protein and mRNA expression under oxidative stress

We determined the effects of miR-1 on the expression of the protein products of Gclc, SOD1, and G6PD by Western blot analysis. The results showed that overexpression of miR-1 had no directly significant effect on the expression of these proteins either in vivo or in vitro. Consistent with previous studies, the expression of redox-related proteins increased significantly under oxidative stress both in vivo (with I/R perfusion) (Fig. 4a) and in vitro (stimulated with H_2O_2)

(Fig. 5a). However, the protective increase in Gclc, SOD1, and G6PD expression under oxidative stress was inhibited by miR-1 overexpression.

We also determined the effects of miR-1 on the mRNA expression of Gclc, SOD1, and G6PD by quantitative real-time RT-PCR analysis. Similar to protein expression, overexpression of miR-1 had no directly significant effects on the expression of these mRNAs under normal control conditions either in vivo or in vitro. In normal control cardiomyocytes, the expression of these mRNAs increased significantly under oxidative stress both in vivo (with I/R perfusion) (Fig. 4b–d) and in vitro (stimulated with H_2O_2) (Fig. 5b–d) (* $P<0.05$). However, the expression of these mRNAs had no significant effects on miR-1 overexpression in cardiomyocytes under oxidative stress either in vivo (with I/R perfusion) or in vitro (stimulated with H_2O_2) (# $P<0.05$ vs. miR-con).

Fig. 2 MiR-1-overexpressed cardiomyocytes exhibit higher ROS levels and lower resistance to oxidative stress. Cardiomyocytes were treated with miR-con, miR-1, miR-1, and AMO-1 or miR-1 and AMO-con for 24 h, and ROS levels were assessed by flow cytometry. **a** Representative results of cardiomyocytes that were treated with miR-con, miR-1, miR-1, and AMO-1 or miR-1 and AMO-con for 24 h and the ROS levels that were detected by flow cytometry analysis. **b** The fluorescence intensity was significantly increased in cardiomyocytes with miR-1

overexpression. AMO-1 decreased the fluorescence intensity significantly in cardiomyocytes with miR-1 overexpression, while AMO-con did not display the effect. Cardiomyocytes were treated with miR-con, miR-1, miR-1, and AMO-1 or miR-1 and AMO-con for 24 h and then treated with H₂O₂ (100 μ M) for 12 h. Representative images of cardiomyocytes before **c** and after **d** treatment with H₂O₂. The cell pictures are magnified by $\times 100$. **e**, **f** Quantification of cell viability. * $P < 0.05$

Discussion

SOD1, Gclc, and G6PD are all redox-related proteins. They play important roles in maintaining the redox balance in the body. Cu/Zn superoxide dismutase (SOD1) is an important scavenger protein that acts against oxidative stress mediated by ROS. Its main function is to catalyze the conversion of superoxide anion to hydrogen peroxide (H₂O₂). Excess H₂O₂ is normally reduced to water by catalase or glutathione peroxidase, preventing the production of hydroxyl radicals. It has been shown that SOD1 plays an important role in suppressing superoxide generation, apoptosis, and the inflammatory response in oxidative stress-related cardiac diseases (Mital et al. 2011; Tanaka et al. 2004; Chen et al. 2000; Land et al. 1994).

Glutamate-cysteine ligase, also known as gamma-glutamylcysteine synthetase, is the rate-limiting enzyme of glutathione (GSH) synthesis. GSH is a tripeptide antioxidant

that plays an important role in maintaining the protein thiol groups, tocopherol, and ascorbate of the cells in their reduced states. Glutathione is synthesized in two adenosine triphosphate-dependent steps as follows: First, gamma-glutamylcysteine is synthesized from L-glutamate and cysteine via the enzyme gamma-glutamylcysteine synthetase. Second, glycine is added to the C-terminal of gamma-glutamylcysteine via the enzyme glutathione synthetase. The first reaction is the rate-limiting step in glutathione synthesis. Animal glutamate cysteine ligase (GCL) is a heterodimeric enzyme composed of a catalytic (Gclc) and modulatory (Gclm) subunit. All the enzymatic activities of the enzyme are contained within Gclc. It has been reported that increased expression of Gclc can upregulate the cellular-reduced glutathione (GSH) level in cardiomyocytes and protect cardiomyocytes from oxidative injury (Zhang et al. 2010; Woo et al. 2008; Wu et al. 2004).

Fig. 3 Verification of SOD1 (a), GclC (b), and G6PD (c) as cognate targets of miR-1 for post-transcriptional repression. Verification of interactions between rat miR-1 and the wild or mutant 3'-UTRs of rat SOD1, GclC, and G6PD in HEK293 cells is determined by luciferase reporter activity. Cells were transfected with the SOD1, GclC, or G6PD construct as indicated. MiR-1 repressed luciferase reporter gene activity with the

WT 3'-UTR of SOD1, GclC, or G6PD. However, miR-1 failed to affect luciferase activity with the MT 3'-UTR of SOD1, GclC, or G6PD. AMO-1 reversed miR-1 induced repression of luciferase reporter gene activity, but AMO-con did not display the effect. Mean±SEM; $n=8$ batches of cells in each group; * $p<0.05$ versus miR-con; # $p<0.05$ versus WT miR-1; unpaired Student's t test

Glucose-6-phosphate dehydrogenase (G6PD) is the rate-limiting enzyme of the pentose phosphate pathway. The pentose phosphate pathway is important for the production of

NADPH and reduced glutathione (G-SH). Reduced NADP (NADPH) is an important free radical deactivator, and G6PD is responsible for maintaining adequate levels of NADPH

Fig. 4 Effect of miR-1 on the mRNA and protein expression of antioxidant proteins in miR-1 transgenic mouse hearts under oxidative stress in vivo (with I/R perfusion). **a** The protein expression of GclC, SOD1, and G6PD in miR-1 transgenic mouse hearts. The protein expression of GclC, SOD1, and G6PD were not affected under normal conditions. I/R perfusion increased these protein expressions, but miR-1 attenuated the increase. Western blot analyses were repeated three times, and this figure is

representative of only one analysis. **b** The mRNA expression of GclC, SOD1, and G6PD in wild-type control mice and in miR-1 transgenic mice after suffering I/R perfusion. The mRNA expressions of GclC, SOD1, and G6PD did not change in miR-1 transgenic mouse hearts after the transgenic mice suffered I/R perfusion. $n=6$ mice in each group; * $P<0.05$ versus WT; # $P<0.05$ versus I/R; unpaired Student's t test

Fig. 5 Effect of miR-1 on the mRNA and protein expression of antioxidant proteins in miR-1-overexpressed rat ventricular cardiomyocytes under oxidative stress in vitro (stimulated with H₂O₂). **a** The expression of Gclc, SOD1, and G6PD at the protein level in neonatal rat ventricular cardiomyocytes. Cardiomyocytes were treated with miR-con, miR-1, or MT miR-1 for 24 h and then treated with H₂O₂ (100 μ M) for 14 h. The protein expression of Gclc, SOD1, and G6PD was then assessed. MiR-1 did not affect the protein expression of Gclc, SOD1, and G6PD, and these protein expressions did not change in miR-1-treated cardiomyocytes after treatment with H₂O₂ (100 μ M) for 14 h. Western blot analyses were

repeated three times, and this figure is representative of only one analysis. **b** The expression of Gclc, SOD1, and G6PD at the mRNA level in neonatal rat ventricular cardiomyocytes treated with miR-con, miR-1, or MT miR-1 for 24 h, followed by treatment with H₂O₂ (100 μ M) for 14 h. The mRNA expressions of Gclc, SOD1, and G6PD did not change in miR-1-treated cardiomyocytes, and these mRNA expressions did not change in miR-1-treated cardiomyocytes after stimulation with H₂O₂. * $P < 0.05$ versus miR-con; # $p < 0.05$ versus H₂O₂+miR-con; unpaired Student's *t* test

inside the cell. G6PD-deficient patients are known to be very sensitive to oxidative stress, exhibiting rapid hemolysis when exposed to infections, certain drugs, and extracorporeal circulation. Studies have highlighted the protective effect of the pentose phosphate pathway for cardiomyocytes in situations of oxidative stress injury. Under conditions of increased oxidative stress, the activity of G6PD is rapidly increased in cardiomyocytes with consequent neutralization of free radical injury (Hecker et al. 2013; Katare et al. 2010; Serpillon et al. 2009; Das et al. 1992).

The expression levels of SOD1, Gclc, and G6PD can be increased by the redox conditions of the internal environment. Increased ROS levels can increase the expression of these redox-related proteins to invoke a compensatory response to fight against oxidative stress. If the reduced systems fail to be regulated by the internal redox condition, especially if they fail to increase their activity when ROS levels increase, the body will suffer from an unbalanced condition. If the unbalanced condition cannot be counteracted in time, the

imbalance will result in some types of disease conditions (Miriya et al. 2012; Shimizu et al. 2010). In the heart, it has been suggested that ROS alter the regulation and organization of collagen in cardiac fibroblasts, resulting in loss of function (Nabeebaccus et al. 2011; Lijnen et al. 2012).

We have reported that miR-1 levels are elevated in individuals with coronary artery disease and rats with experimental myocardial infarction, both of which are oxidative stress-related conditions. We also found that cardiac function decreased significantly in miR-1-overexpressed mice. Database screening indicated that miR-1 has potential complementary sites with oxidative stress-related proteins Gclc, SOD, and G6PD. Previous studies have indicated that miRNA-binding sites are transferable and sufficient to confer miRNA-dependent gene silencing. We inserted the 3'-UTRs of Gclc, SOD1, and G6PD into the 3'-UTR of a luciferase reporter plasmid containing a constitutively active promoter to determine the effects of miR-1 on reporter expression. Cotransfection of miR-1 with the plasmid into

HEK293 cells consistently produced less luciferase activity, whereas the mutant miR-1 did not reduce luciferase activity. Transfection of AMO-1 into the cells eliminated the silencing effects of miR-1 on the activities of the WT Gclc, SOD1, and G6PD luciferase chimeric vector and target sequences. The same results were obtained with both rat and human miR-1 and the 3'-UTRs of Gclc, SOD1, and G6PD.

We measured the mRNA and protein levels of Gclc, SOD1, and G6PD under normal and oxidative stress conditions. With miR-1 treatment, these antioxidant enzymes were not induced in hearts subjected to ischemia-reperfusion injury, nor were they induced in neonatal rat ventricular cardiomyocytes treated with high concentrations of H₂O₂. Interestingly, these enzymes were not inhibited by miR-1 at basal levels. A possible explanation for this result is that internal redox status is maintained by an orchestrated regulatory network, as well as that anti-oxidative enzymes play important roles in defense only when induced by oxidative stress. Overexpression of miR-1 did not affect the target mRNA and protein expression levels under normal condition. In response to diseases and other kinds of stress, the concentration of reactive oxygen species is increased by enhanced metabolism. Correspondingly, antioxidant capacity is also elevated so that the oxidation-reduction balance is maintained. However, miR-1 reduced the protein levels of Gclc, SOD1, and G6PD under oxidative stress conditions.

Many studies have shown that oxidative stress is one of the mechanisms that determine cardiomyocyte apoptosis versus survival, and miR-1 is involved in oxidative stress-related cardiovascular diseases. MiR-1 is involved in regulating Hsp60 expression, contributing to high glucose-mediated apoptosis in cardiomyocytes (Shan et al. 2010). Induced pluripotent stem cells treated with H₂O₂ showed increases in miR-1 expression and decreases in IGF-1, which is a target of miR-1 and inhibits H₂O₂-induced mitochondrial dysfunction, cytochrome-c release, and apoptosis (Li et al. 2012). Overexpression of miR-1 increased ROS and decreased cell viability. Insulin decreased miR-1 expression and induced a markedly protective effect on miR-1-induced injury under oxidative stress, which may be mediated by the Akt-mediated pathway (Chen et al. 2012).

Our results suggest that post-transcriptional repression of SOD1, Gclc, and G6PD by miR-1 can contribute to increased ROS levels and susceptibility to oxidative stress in the hearts of miR-1 transgenic mice. An improved understanding of miR-1 in heart tissue would facilitate the design of novel strategies for cardioprotection against oxidative stress.

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