MicroRNA-103/107 Regulate Programmed Necrosis and Myocardial Ischemia/Reperfusion Injury Through Targeting FADD

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Rationale: Necrosis is one of the main forms of cardiomyocyte death in heart disease. Recent studies have demonstrated that certain types of necrosis are regulated and programmed dependent on the activation of receptor-interacting serine/threonine-protein kinase (RIPK) 1 and 3 which may be negatively regulated by Fas-associated death domain protein (FADD). In addition, microRNAs and long noncoding RNAs have been shown to play important roles in various biological processes recently.

Objective: The purpose of this study was to test the hypothesis that microRNA-103/107 and H19 can participate in the regulation of RIPK1- and RIPK3-dependent necrosis in fetal cardiomyocyte-derived H9c2 cells and myocardial infarction through targeting FADD.

Methods and Results: Our results show that FADD participates in H$_2$O$_2$-induced necrosis by influencing the formation of RIPK1 and RIPK3 complexes in H9c2 cells. We further demonstrate that miR-103/107 target FADD directly. Knockdown of miR-103/107 antagonizes necrosis in the cellular model and also myocardial infarction in a mouse ischemia/reperfusion model. The miR-103/107-FADD pathway does not participate in tumor necrosis factor-α-induced necrosis. In exploring the molecular mechanism by which miR-103/107 are regulated, we show that long noncoding RNA H19 directly binds to miR-103/107 and regulates FADD expression and necrosis.

Conclusions: Our results reveal a novel myocardial necrosis regulation model, which is composed of H19, miR-103/107, and FADD. Modulation of their levels may provide a new approach for preventing myocardial necrosis. (Circ Res. 2015;117:352-363. DOI: 10.1161/CIRCRESAHA.117.305781.)

Key Words: Fas-associated death domain protein ■ H19 long noncoding RNA ■ ischemia/reperfusion injury ■ miRNA ■ myocardial infarction ■ necrosis ■ oxidative stress

Numerous studies have demonstrated that cell death is an important component in the pathogenesis of various cardiac diseases, including heart failure, myocardial infarction (MI) and ischemia/reperfusion (I/R). Myocytes injured during heart disease include both apoptotic and necrotic cells. Studies have shown that necrosis is most prominent in the failing heart and I/R heart, indicating that necrosis plays an important role in the pathological process of cardiac disease. Nevertheless, the underlying mechanisms of cardiomyocyte death are not fully understood.

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Necrosis has long been considered to be passive and accidental. However, a growing body of evidence has refuted this view and revealed the existence of multiple pathways regulating necrosis. Death receptors have been shown to induce a particular type of necrotic death in certain cell types. This process is referred to as programmed necrosis or necroptosis and is mediated by the receptor-interacting serine/threonine-protein kinase (RIPK) 1 and 3. Necroptosis initiated by tumor necrosis factor (TNF)-α and its receptor (TNFFR) 1 has been most extensively characterized. On death receptor ligation, RIPK1 and RIPK3 are catalytically activated by phosphorylation and interact via their RIP homotypic interaction motif RHIM. Necrostatin-1, a pharmacological inhibitor of RIPK1 kinase, seems to inhibit RIPK1–RIPK3 interaction as well as inhibit necrotic cell death. Although growing evidence suggests that necrosis is regulated, the molecular...
mechanisms of programmed necrosis are unclear. The molecules and pathways of necroptosis require further study and could have clinical implications.

Fas-associated protein with death domain (FADD) participates in death-inducing signaling complex formation and induces apoptosis through binding to the intracellular death domain of death receptors in the TNF family, such as Fas and TNFR1. In addition to inducing extrinsic apoptosis, FADD is known to suppress necroptosis under various conditions. Inhibition of FADD sensitizes cells to necroptosis induction after death receptor stimulation. Mice lacking FADD show embryonic lethality as a result of cardiac abnormalities. The lethality of FADD KO mice is successfully rescued by RIPK1 deletion. Another study reported that knockdown of FADD resulted in an increase in RIPK1–RIPK3 complex formation and necrosis in the presence of caspase inhibitor. These studies suggest negative roles for FADD in RIPK1- and RIPK3-dependent necroptosis. However, the molecular mechanism by which FADD participates in the necrotic machinery has not been elucidated. Furthermore, the physiological and pathologic significance of the mechanisms of FADD in regulating programmed necrosis in the heart also remains to be revealed.

MicroRNAs (miRNAs) are a class of small noncoding RNAs and that negatively regulate target genes. MiRNAs are involved in cardiac events such as the conductance of electric signals, muscle contraction, heart growth, and morphogenesis. Mounting evidence shows that miRNAs are involved in the pathogenesis of cardiac diseases such as MI and heart failure. Therefore, it might be possible to manipulate miRNAs to achieve therapeutic effects. However, the role of miRNAs in the regulation of necroptosis and how they target FADD in the heart are unknown. Other noncoding RNAs especially the long noncoding RNAs (lncRNAs) also play important roles in biological processes. LncRNAs participate in a variety of biological processes, such as epigenetic control of chromatin, maintenance of nuclear body structure, and RNA splicing. Because lncRNAs play an essential role in biological processes, it is possible that they also regulate oxidative stress–induced necrosis.

Taking all these into consideration, the present study aims to identify molecules that can regulate cardiomyocyte necrosis, and our results reveal a model that is composed of lncRNA H19, miR-103/107, and FADD.

### Methods

A detailed account of the Materials and Methods used in this study is provided in the Online Data Supplement.

### Results

#### FADD Can Inhibit H2O2-Induced Necrotic Cell Death in H9c2 Cells

H2O2 is well known to be an important factor inducing both apoptosis and necrosis. Its role depends on the exact concentration applied and the cell type tested. We tested the effect of H2O2 on the death of the heart cell line H9c2. We observed that treatment with 100 μmol/L H2O2, mainly triggered apoptosis, whereas the concentration of 500 μmol/L H2O2 preferentially caused necrosis (Online Figure IA). A time-dependent increase in the apoptotic cells was observed in H9c2 on treatment with 100 μmol/L H2O2 (Figure 1A). In H9c2 cells subjected to 500 μmol/L H2O2, a time-dependent increase in the necrotic cells was observed (Figure 1B). Apoptosis and necrosis were further assessed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay and propidium iodide exclusion (Figure 1C). Necrosis was also determined by lactate dehydrogenase release assay and the findings were the same as above (Online Figure IB).

To further confirm these results, we used electron microscopy to detect the morphological hallmarks of H9c2 cells (Online Figure IC). Taken together, these data indicate that a low concentration of H2O2 induces apoptosis, whereas a high concentration of H2O2 induces necrosis among H9c2 cells. Administration of necroptosis inhibitor-necrostatin-1 led to a reduction in necrosis induced by H2O2 (Online Figure ID).

The importance of FADD in necrosis has recently been reported. Thus, we investigated whether FADD participated in the H2O2-induced necrotic program. Both protein and mRNA levels of FADD were not changed significantly in H9c2 cells exposed to 100 μmol/L H2O2 (Figure 1D). However, exposure to 500 μmol/L H2O2 substantially downregulated the protein level of FADD in a time-dependent manner, whereas the mRNA level of FADD was not changed (Figure 1E). These results indicate that FADD may participate in the H2O2-induced necrotic program. Knockdown of endogenous FADD sensitized cells to undergo necrosis in H9c2 cells exposed to 100 μmol/L H2O2 for 12 hours (Online Figure IE) or 24 hours (Figure 1F) and apoptotic cells were decreased significantly (Online Figure IF).

Enforced expression of FADD attenuated necrotic cell death induced by 500 μmol/L H2O2 (Figure 1G; Online Figure IG), whereas there was no significant change in apoptosis (Online Figure IH). These results were also confirmed by lactate dehydrogenase release assay (Online Figures IJ and II). Thus, these results indicate that FADD participates in the maintenance of cardiomyocyte survival by inhibiting necrosis.

#### FADD Regulates Necrotic Cell Death Through Influencing the RIPK1/RIPK3 Complex

The formation of a complex containing RIPK1 and RIPK3 is crucial to the process of necrosis. The expression levels of RIPK1 and RIPK3 were decreased in H9c2 cells exposed to 100 μmol/L H2O2 (Online Figure IIA) and increased in H9c2 cells exposed to 500 μmol/L H2O2 (Online Figure IIB). It previously has been shown that both RIPK1 and RIPK3 can be degenerated by activated caspase-8, which may explain why RIPK1 and RIPK3 are downregulated under low concentration of H2O2. FADD has been demonstrated to bind to

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>FADD</td>
<td>Fas-associated protein with death domain</td>
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<tr>
<td>I/R</td>
<td>ischemia/reperfusion</td>
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<tr>
<td>IncRNAs</td>
<td>long noncoding RNAs</td>
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<tr>
<td>MI</td>
<td>myocardial infarction</td>
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<tr>
<td>miRNA</td>
<td>microRNA</td>
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<tr>
<td>RIPK</td>
<td>receptor-interacting serine/threonine-protein kinase</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
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<td>TNFR1</td>
<td>tumor necrosis factor-α receptor 1</td>
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the RIPK1/RIPK3 complex. This led us to consider the possibility that FADD may inhibit necrosis through RIPK1 and RIPK3 in H9c2 cells exposed to H$_2$O$_2$. We first tested the role of RIPK1 and RIPK3 in H$_2$O$_2$-induced H9c2 cell necrosis. Knockdown of RIPK1 (Online Figure IIC) or RIPK3 (Online Figure IID) attenuated necrotic cell death induced by H$_2$O$_2$ (Figure 2A and 2B). The results suggested that RIPK1 interacted with RIPK3 in H9c2 cells exposed to 500 μmol/L H$_2$O$_2$ (Figure 2C). Necrostatin-1 could inhibit the formation of the RIPK1/RIPK3 complex, which suggested that the activity of RIPK1 is essential for the binding of RIPK1 to RIPK3 (Online Figure IIE).

We also tested the role of FADD participated in necrosis dependent on RIPK1 or RIPK3. Knockdown of FADD promoted low-dose H$_2$O$_2$-induced necrotic cell death. Administration of RIPK1 small interfering RNA or RIPK3 small interfering RNA reduced FADD knockdown-enhanced sensitivity of necrotic cell death (Figure 2D and 2E). We then tested the ability of FADD to influence the binding of RIPK1 and RIPK3. A strong association between FADD and RIPK1 could be observed in cells without treatment, but the association became weaker when cells were exposed to H$_2$O$_2$ (Figure 2F). Enhanced expression of FADD led to significant reduction in the RIPK1 and RIPK3 association levels (Figure 2G), whereas knockdown

**Figure 1.** Fas-associated protein with death domain (FADD) can inhibit H$_2$O$_2$-induced necrosis. A to C, H$_2$O$_2$ induces apoptosis and necrosis in H9c2 cells treated with 100 μmol/L (A) or 500 μmol/L H$_2$O$_2$ (B) at the indicated time. Apoptotic and necrotic cells were analyzed by flow cytometry using Annexin V/propidium iodide (PI) assay. C, Apoptosis and necrosis were detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) and PI assay. Representative images show TUNEL-positive or PI-positive cells (left). Green, TUNEL-positive nuclei; red, PI-positive nuclei; blue, 4′,6-diamidino-2-phenylindole–stained nuclei. The quantitative analysis of cell death is shown (right). *P<0.05 vs control. D and E, FADD protein and mRNA levels were detected by immunoblot or quantitative reverse transcriptase polymerase chain reaction in H9c2 cells treated with 100 μmol/L H$_2$O$_2$ (D) or 500 μmol/L H$_2$O$_2$ (E) at the indicated time. F, Knockdown of FADD using its small interfering RNA (siRNA) increased H$_2$O$_2$-induced necrosis in H9c2 cells exposed to 100 μmol/L H$_2$O$_2$ for 24 hours, compared with its scramble form (FADD-sc). Necrosis was detected by PI assay. FADD levels were detected by immunoblot. G, Enforced expression of FADD prevented H$_2$O$_2$-induced necrosis in H9c2 cells exposed to 500 μmol/L H$_2$O$_2$ for 24 hours. *P<0.05 vs H$_2$O$_2$ alone.
FADD promoted the binding of RIPK1 to RIPK3 (Figure 2H). TNFRSF1A-associated via death domain is another component of death-inducing signaling complex.11 We tested whether TNFRSF1A-associated via death domain can influence the formation of RIPK1/RIPK3 complex. Knockdown TNFRSF1A-associated via death domain did not promote the formation of RIPK1/RIPK3 complex (Online Figure IIIF). We also detected the subcellular localization of RIPK1/RIPK3 complex. RIPK1/ RIPK3 complex could be detected in both cytosol and mitochondria (Online Figure IIIG). These data indicate that FADD inhibits necrosis by disturbing the formation of RIPK1/RIPK3 complex via binding to RIPK1.

It has been suggested that RIPK1 and RIPK3 also participate in the regulation of apoptosis.26,27 Knockdown of RIPK1 or RIPK3 attenuated apoptotic cell death induced by low-dose H2O2 (Online Figures IIIH and IIII). The mechanism by which RIPK1 and RIPK3 regulate apoptosis requires further research.

**MiR-103/107 Participate in the Regulation of FADD Expression**

To explore the underlying mechanism by which FADD was downregulated by H2O2, we tested the ability of miRNA to control FADD expression. We analyzed the 3' untranslated region sequence of FADD using RNA hybrid approach. No
potential miRNA binding sites were found. Because miRNAs can also bind to the coding region of target gene, we analyzed the coding sequence of FADD and observed a region that is complementary to miR-103/107 (Figure 3A). We then attempted to evaluate whether miR-103/107 modulated FADD expression. Enforced expression of miR-103/107 resulted in a significant reduction of FADD protein but not mRNA levels in H9c2 cells (Figure 3B). In contrast, administration of miR-103/107 antagomir which could knockdown both miR-103 and miR-107 resulted in an increase in FADD protein levels (Figure 3C). These findings suggest that miR-103/107 modulate FADD expression at post-transcriptional level.

To verify whether miR-103/107 directly target FADD, we cloned FADD coding sequence containing miR-103/107 binding site downstream of the luciferase reporter gene (FADD BS-Wt). MiR-103/107 induced a decrease in the luciferase activity (Figure 3D). We also generated a mutated luciferase construct (FADD BS-Mut), in which mutations were introduced into the miR-103/107 binding site (Online Figure IIIA). The introduction of mutation substantially reduced the inhibitory effect of miR-103/107 (Figure 3D). To further investigate the effect of miR-103/107 on FADD regulation, we cloned FADD cDNA upstream of green fluorescent protein (GFP) (FADD-GFP) and examined GFP expression driven by the binding site of FADD (Online Figure IIIB). MiR-103/107 overexpression induced a decrease in the fluorescence intensity (Figure 3E) and protein levels (Figure 3F) of GFP. Pull down of biotin-labeled miR-103/107 was also performed and the result showed both miR-103 and miR-107 could bind to FADD coding sequence region (Online Figure IIIC). Thus, our data indicate that miR-103/107 are able to target FADD directly.
MiR-103/107 Regulate H₂O₂-Induced Necrosis Through FADD

Subsequently, we tested whether miR-103/107 have a functional role in H9c2 cells necrosis. When exposed to 100 μmol/L H₂O₂, both miR-103 and miR-107 levels were not changed obviously (Figure 4A). However, exposure to 500 μmol/L H₂O₂ markedly elevated miR-103 and miR-107 levels in H9c2 cells (Figure 4B).

We next explored the role of miR-103/107 in cardiomyocyte necrosis. Knockdown of endogenous miR-103/107 diminished high-dose H₂O₂-induced necrosis (Figure 4C), but did not influence low-dose H₂O₂-induced necrosis (Online Figure IVA). Cotransfection of miR-103 and miR-107 alone had no significant effect on necrosis (Online Figure IVB), whereas enforced miR-103 or miR-107 expression sensitized H9c2 cells to necrosis induced by H₂O₂ (Figure 4D and Online Figure IVC). We wondered whether miR-103/107 regulated necrosis by targeting FADD. Administration of miR-103/107 antagonir inhibited necrotic cell death, but this inhibitory effect was significantly abolished by FADD knockdown (Figure 4E). Overexpression of miR-103 promoted low-dose H₂O₂-induced necrotic cell death. FADD showed a strong inhibitory effect on necrotic cell death in the presence of miR-103 (Figure 4F). Furthermore, RIPK3 knockdown also showed a strong inhibitory effect on necrotic cell death in the presence of miR-103 (Figure 4G). These results reveal that miR-103/107 contribute to necrotic cell death by targeting FADD.

Caveolin-1 is a target of miR-103/107 and plays a significant role in cardiovascular disease and dysfunction. Caveolin-1 expression was decreased in H9c2 cells exposed to 500 μmol/L H₂O₂ (Online Figure IVD). Knockdown miR-103/107 attenuated the decrease of caveolin-1 induced by H₂O₂ (Online Figure IVE). These results suggest that caveolin-1 may be another target of miR-103/107 in H₂O₂-induced necrotic program.

MiR-103/107 Are Not Involved in TNF-α-Induced Necrosis

TNF-α is the most extensively studied extracellular signal that leads to apoptosis and necroptosis. Apoptosis resulting from...
activation of death receptors can be completely rescued by caspase inhibitors, such as carboxbenzoxyl-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (z-VAD-fmk). However, in some cell types, the same caspase inhibitor switches the death program to necrosis in response to TNF-α. This form of cell death is dependent on the kinase activities of RIPK1 and RIPK3. MiR-103/107 participated in H₂O₂-induced necrosis, which led us to consider whether both miRNAs are involved in TNF-α-induced necrosis. TNF-α alone could induce apoptosis in H9c2 cells (Figure 5A). The number of necrotic cells was increased dramatically in H9c2 cells on combinatorial treatment with TNF-α and z-VAD-fmk (Figure 5B). TNF-α alone or combination with z-VAD-fmk did not affect the expression levels of miR-103/107 (Figure 5C) and FADD (Figure 5D). Thus, our data indicate that miR-103/107-FADD pathway may not be involved in TNF-α–induced necrosis. Subsequently, we tested whether TNFR signaling activation was essential for H₂O₂-induced necrosis. We generated small interfering RNA constructs for TNFR1 (Online Figure V). H₂O₂-induced necrosis was not affected by TNFR1 knockdown (Figure 5E). Taken together, these results suggest that the activation of kinase activity of RIPK1 and RIPK3 may

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**Figure 6. MicroRNA (MiR)-103/107 regulate necrotic cell death in the heart.**

A and C, MiR-103, miR-107 and Fas-associated protein with death domain (FADD) levels in the myocardium administrated to ischemic injury. Mice were subjected to cardiac ischemia at the indicated time. Expression levels of miR-103 and miR-107 were detected in ischemic zone (A) or border zone (B). *P<0.05 vs sham. C, The blots showed the representative protein levels of FADD (n=8). D, Knockdown of miR-103/107 attenuated myocardial necrosis on ischemia/reperfusion (I/R). Anta-103/107 or Anta-NC was injected into the mice. I/R was performed 24 hours after the last injection. Propidium iodide (PI) was injected into the mice to label necrotic cells 1 hour before I/R. Mice were subjected to 45 minutes of LAD ligation followed by 1 hour of reperfusion. Representative images of ventricular myocardium sections are shown (left) and the quantitative analysis of PI-positive cells is shown (right). Red, PI-positive myocyte nuclei; blue, 4′,6-diamidino-2-phenylindole–stained nuclei (n=8). Scale bar, 20 μm. *P<0.05 vs I/R alone. E, Myocardial infarct sizes were measured 24 hours after reperfusion. The ratios of area at risk (AAR) to left ventricular (LV) area and infarct (INF) area to AAR are shown (n=8). *P<0.05 vs I/R alone. F and G, The levels of interleukin (IL)-1β and tumor necrosis factor-α (TNF-α) were analyzed in mice I/R heart tissues by quantitative reverse transcriptase polymerase chain reaction. Mice were treated as described in D. *P<0.05 vs I/R alone. H, Knockdown of miR-103/107 preserves cardiac function on I/R. Echocardiographic analysis was performed as described in the Online Methods. FS indicates fractional shortening of left ventricular diameter; LVIDd, diastolic left ventricular internal diameters; and LVIDs, systolic left ventricular internal diameters (n=8). *P<0.05 vs I/R alone.
not depend on the activation of receptors on the cell surface in H₂O₂-induced necrosis.

**MiR-103/107 Regulate Necrotic Cell Death in the Heart**

Having demonstrated that miR-103/107 are involved in H₂O₂-induced necrosis by targeting FADD in vitro, we further investigated the role of miR-103/107 and FADD in the pathogenesis of cardiac infarction in a mouse model of I/R. I/R has been shown to induce ROS production and necrosis in the heart. Both miR-103 and miR-107 were elevated in the ischemic zone (Figure 6A) but not in the border zone (Figure 6B) of ischemic heart. Protein levels of FADD in ischemic zone of heart were decreased (Figure 6C). MiR-103/107 antagonir (Anta-103/107) or antagonim control (Anta-NC) were injected into mice to knockdown endogenous miR-103/107 expression level in heart (Online Figure VIA). I/R led to a reduction in FADD, and this reduction was attenuated by miR-103/107 knockdow (Online Figure VIB). The administration of miR-103/107 antagonim resulted in a reduction in the myocardial necrosis (Figure 6D), reduced myocardial infarct sizes (Figure 6E), and reduced plasma levels of cardiac necrosis biomarker troponin T (Online Figure VIC), but not affect myocardial apoptosis as analyzed by TUNEL assay (Online Figure VID). Necrosis promotes inflammation and as expected inflammatory factors, TNF-α, interleukin-1β were detected. I/R led to an upregulation of TNF-α and interleukin-1β, and this upregulation was attenuated by miR-103/107 knockdow (Figure 6F and 6G). Cardiac fibrosis revealed that increased collagen deposition was also reduced in the Anta-103/107 group (Online Figure VIE) and the cardiac function was ameliorated by knockdow of miR-103/107 (Figure 6H). Thus, miR-103 and miR-107 may have a significant role in myocardial necrosis and MI.
H19 Interacts With miR-103/107 and Regulates miR-103/107 Expression

The mechanism by which \( \text{H}_2\text{O}_2 \) and I/R downregulate miR-103/107 is unknown. Recent studies have suggested that IncRNAs may act as endogenous sponge RNA to interact with miRNAs and influence the expression of miRNAs. We tested whether IncRNAs can interact with miR-103/107. We compared the sequences of IncRNAs with that of miR-103 and miR-107 using the bioinformatics program RNA hybrid and found some potential IncRNAs containing target sites of miR-103/107. Among several IncRNAs (Online Figure VIIA), H19 was substantially reduced in H9c2 cells exposed to 500 \( \mu \text{mol/L} \) \( \text{H}_2\text{O}_2 \) (Figure 7A) but was not changed in H9c2 cells exposed to 100 \( \mu \text{mol/L} \) \( \text{H}_2\text{O}_2 \) (Figure 7B). There are 3 potential miR-103/107 binding sites in H19 (Online Figure VIIIB). H19 was also reduced in ischemic hearts in animal model (Online Figure VICH).

We tested whether H19 can regulate miR-103/107 expression. Enforced expression of H19 (Online Figure VIIID) led to a reduction in both miR-103 and miR-107 levels (Figure 7C and 7D). MiR-103 and miR-107 levels were elevated in cells on knockdown of endogenous H19 (Online Figure VIIIE; Figure 7E and 7F). \( \text{H}_2\text{O}_2 \)-induced upregulation of miR-103 and miR-107 was attenuated by H19 (Online Figure VIIIF; Figure 7G and 7H).

We then used a biotin–avidin pull-down system to test whether miR-103 and miR-107 can directly bind to H19. H19 was pulled down by biotinylated miR-103/107, but not mutant miR-103/107 (Figure 7I). The introduction of mutations in the seed sequence disrupts base pairing between H19 and miR-103/107 (Online Figure VIIIG) indicating that the recognition of miR-103/107 by H19 is in a sequence-specific manner. Other potential IncRNAs were not pulled down by miR-103 or miR-107 (Online Figure VIIH). We also used an inverse pull-down assay to test whether H19 can pull down miR-103 and miR-107. The results showed that both miRNAs could be coprecipitated by biotinylated H19 but not by biotinylated control (Figure 7J). These data suggest that H19 is able to directly bind to miR-103/107 and regulate miR-103/107 levels.

Figure 8. H19 regulates necrosis through targeting microRNA (miR)-103/107 and Fas-associated protein with death domain (FADD). A to D, H19 can regulate FADD. FADD protein levels were detected by immunoblot. Quantitative analysis of FADD levels is shown in the lower panels. A, H9c2 cells were transfected with H19-small interfering RNA (siRNA) or H19-scrambled form (sc). B, H9c2 cells were infected with adenoviral H19 or H19-sc. C and D, H9c2 cells were transfected with miR-103 mimic (C) or miR-107 mimic (D), and then infected with H19 or β-gal. *P<0.05 vs control. C and D, H9c2 cells were transfected with miR-103 or miR-107 alone or miR-107 alone. E, H19 counteracts the inhibitory effect of miR-103/107 on FADD. Human embryonic kidney 293 (HEK-293) cells were transfected with miR-103 or miR-107 or H19 or empty control (Figure 7C). FADD protein levels were detected by immunoblot. *P<0.05 vs miR-103 alone or miR-107 alone. H19 attenuates decrease of FADD and necrosis induced by H2O2. H9c2 cells were infected with H19 or β-gal and exposed to 500 \( \mu \text{mol/L} \) \( \text{H}_2\text{O}_2 \). *P<0.05 vs H2O2 alone. F and G, Knockdown H19 using its siRNA increases necrosis in H9c2 cells induced by 100 \( \mu \text{mol/L} \) \( \text{H}_2\text{O}_2 \). *P<0.05 vs \( \text{H}_2\text{O}_2 \) alone. H and I, H19-decreased necrosis was abolished by miR-103/107 (H) or FADD-siRNA in H9c2 cells induced by \( \text{H}_2\text{O}_2 \). *P<0.05 vs H19 treated with \( \text{H}_2\text{O}_2 \).
H19 Regulates Necrosis by Targeting miR-103/107 and FADD

H19 is highly expressed in the heart under physiological conditions. However, the function of H19 in the heart is unknown. The ability of H19 to interact with miR-103/107 led us to study whether H19 is able to regulate necrosis through miR-103/107 and FADD. Knockdown of H19 reduced FADD levels (Figure 8A). Overexpression of H19 resulted in the upregulation of FADD expression levels (Figure 8B). H19 counteracted the effect of miR-103 and miR-107 on FADD expression (Figure 8C and 8D). A luciferase reporter assay showed that H19 attenuated the inhibitory effect of miR-103 and miR-107 on FADD binding site (Figure 8E).

Next, we examined the effect of H19 on necrosis. Enforced expression of H19 inhibited necrosis and downregulation of FADD induced by H2O2 (Figure 8F). Knockdown of H19 significantly increased the sensitivity of H2O2-induced necrosis (Figure 8G). MiR-103/107 attenuated the inhibitory effect of H19 on necrosis (Figure 8H), and a similar result was observed in the presence of the FADD small interfering RNA (Figure 8I). These results indicate that H19 mediates necrotic cell death in cardiomyocytes through modulating miR-103/107-FADD signaling pathway.

Whether H19 is involved in TNF-α–induced necrosis? TNF-α alone or combination with z-VAD-fmk did not affect the expression level of H19 (Online Figure VIIIA). Knockdown of H19 promoted the death program to necrosis in response to TNF-α (Online Figure VIIIB). Thus, our data indicate that H19 may play a role in cardiac protection through inhibiting cardiomyocyte death induced by a variety of stimulation.

Discussion

Necrosis is one of the main forms of cell death in heart disease. To suppress myocardial necrosis and the consequent heart disease, it is essential to elucidate the underlying molecular mechanisms and discover impactful therapeutic target. Our present work demonstrated that knockdown of miR-103/107 attenuated necrosis in H2O2-treated H9c2 cells and I/R injury in an animal model. We found that FADD was negatively regulated by miR-103/107. FADD acted as a negative regulator of necrosis by preventing the formation of RIPK1–RIPK3 complex. Moreover, we identified that H19 inhibited myocardial necrosis by downregulating miR-103/107. Our results reveal a novel mechanism regulating myocardial necrosis.

MiR-103/107 are abundant in the brain and lung, but in the heart both miRNAs are expressed at low level. Our data showed that miR-103/107 were upregulated in H9c2 cells exposed to H2O2 and in I/R heart. However, the function of miR-103/107 in heart is unclear. In this study, we demonstrated that miR-103/107 participated in necrosis and MI through targeting FADD. MiR-103 and miR-107 belong to the miR-103/107 family. They have overlapping targets and similar functions. Studies demonstrated that miR-103/107 are involved in numerous biological processes including cell division, cell differentiation, angiogenesis, and insulin sensitivity. Whether miR-103/107 are involved in necrosis in other tissues or diseases such as cancer and neurodegenerative diseases is an interesting question for future investigation. Only a few miRNAs such as miR-874 and miR-155 have been found to be involved in necrosis. Other miRNAs participating in necrosis need to be further explored in future studies.

TNF-α is the most studied extracellular signaling protein that leads to necroptosis. Insights into the molecular mechanism of necrosis were obtained largely from studies in the context of TNFR1 engagement. In the present study, we found that TNFR1 is not required for H2O2-induced necrosis. Therefore, the activation of RIPK1 and RIPK3 may be independent of TNF-α–TNFR1 pathway. Other death receptors have been reported to induce necrotic cell death on activation of RIPK1 and RIPK3, such as Fas, TNFR2, TRAILR1 and TRAILR2. Whether these death receptors participate in H2O2-induced necrosis needs to be further explored. In addition, studies demonstrated that other triggers induce programmed necrosis in a death receptor–independent manner, such as virus, NO, and chemical stress. Interferon regulation of necrosis via interferon-induced RIPK1–RIPK3 complex formation required the RNA-responsive kinase PKR in a death receptor–independent manner. PKR interacts with RIPK1 to initiate the formation of RIPK1–RIPK3 complex and trigger necrosis. H2O2-induced necrosis may also be independent of death receptor. Studies found that RIPK1 and RIPK3 are activated in ischemic heart. The activation and formation of the RIPK1–RIPK3 complex are involved in a complicated mechanism initiated by different triggers, especially under pathological conditions such as cardiac I/R and needs to be further explored.

FADD has been shown to negatively regulate necrosis under various conditions, but the mechanism by which FADD license necrosis under pathological conditions is not fully understood. We demonstrated that RIPK1 is a FADD-interacting partner under normal condition in H9c2 cells. After the treatment with H2O2, the expression level of FADD was downregulated and the RIPK1–RIPK3 complex was formed. However, the formation of RIPK1–RIPK3 complex and necrosis were attenuated by enhanced expression of FADD under the same condition. We speculate that the mechanism by which FADD prevents RIPK-driven necrosis is by physically associating with RIPK1 to prevent it from interacting with RIPK3. Furthermore, we demonstrated that FADD was negatively regulated by miR-103/107. Recent studies have shown that FADD could be regulated through other forms. It was reported that ubiquitination and degradation of FADD mediated by Makorin Ring Finger Protein 1 E3 ligase regulate death receptor–mediated necroptosis. Another study showed that phosphorylation as a mechanism that disabled FADD and facilitated interferon-induced necrosis. The regulation of FADD in necrosis may be cell type-dependent as well as stimulation dependent.

Necrosis is recognized as a cause for inflammation. Necrotic cells that have undergone plasma membrane permeabilization release a variety of proteins and nucleic acids that not only are markers of membrane dysfunction but also mediate the inflammatory response. For example, the release of the histone-associated protein high-mobility group protein B1 stimulates inflammation through multiple mechanisms involving Toll-like receptors. Inflammation and the subsequent reaction are considered to be a major cause of MI. For instance, studies found that inhibition of RIPK1 by adding necrostatin-1 inhibits RIPK1-dependent necrosis, reduces infarct size, and decreases inflammatory influx. It has been previously shown that deficiency of FADD induces severe skin and chronic intestinal disease.

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inflammation triggered by RIPK3-mediated programmed necrosis. We speculate that miR-103/107-mediated downregulation of FADD could participate in the inflammatory response in I/R heart. Our present study reveals that knockdown of miR-103/107 decreases the expression levels of inflammatory cytokines TNF-α and interleukin-1β. However, the mechanisms of inflammation triggered by necrotic cardiomyocytes are not clear and need to be further investigated. Modulation of the mechanism may provide a new approach for preventing MI.

It has been shown that certain IncRNAs can competitively inhibit the function of miRNAs by base-pairing with miRNAs, analogous to how an artificial miRNA sponge function.60 Our results show that the expression of miR-103/107 was reduced by the sponge H19. Similar to our results, H19 has been found to be a molecular sponge for the major let-7 family of miRNAs, which are known to play important roles in diverse physiological and pathological processes.61 It is known that H19 was highly expressed in the developing embryo, whereas its expression is downregulated after birth, except in cardiac and skeletal muscle.62,63 This characteristic of expression implies potential functions in heart development and functional maintenance. However, how H19 performs its function in the heart remains largely unknown. Recent studies have revealed that IncRNAs played an important role in the heart development and cardiac disease.64,65 Our present work reveals a novel function of H19 in regulating H9c2 cell necrosis. H19 serves as a sponge of miR-103/107 regulating the expression of FADD, which attenuates necrotic responses. The binding sites of miR-103/107 in H19 are conserved across species. This indicates that H19 may have a similar function in humans. Our results provide a new clue for the understanding of IncRNAs-controlled cellular events.

In summary, our data reveal links among H19, miR-103/107, FADD, and RIPK1/RIPK3 in the necrotic program of heart cells. Future studies are needed to elucidate how this pathway is integrated into the complex necrotic cascade and its relationship with other necrotic factors. Our results suggest that the modulation of miR-103/107 may represent a therapeutic approach to treat necrosis-related cardiac disease, including MI.

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Disclosures
None.

References

Disclosures
None.

References
Long noncoding RNA H19 acts as an endogenous sponge RNA to MiR-103/107 family participates in H2O2-induced cardiomyocyte necrosis and myocardial infarction through downregulating FADD. MiR-103/107 and MiR-185 can induce cell cycle arrest in human non small cell lung cancer cell lines. The murine H19 gene is activated during embryonic stem cell differentiation in vitro and at the time of implantation in the developing embryo. The murine H19 gene is activated during embryonic stem cell differentiation in vitro and at the time of implantation in the developing embryo. What New Information Does This Article Contribute? What Is Known? What Is New? No new information does this article contribute.
MicroRNA-103/107 Regulate Programmed Necrosis and Myocardial Ischemia/Reperfusion Injury Through Targeting FADD
Jian-Xun Wang, Xiao-Jie Zhang, Qian Li, Kun Wang, Yin Wang, Jian-Qin Jiao, Chang Feng, Sun Teng, Lu-Yu Zhou, Ying Gong, Zhi-Xia Zhou, Jia Liu, Jian-Ling Wang and Pei-feng Li

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SUPPLEMENTAL MATERIAL

DETAILED METHODS

Cell culture and treatment
Fetal cardiomyocyte-derived H9c2 cell line (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin and 110mg ml⁻¹ sodium pyruvate in a humidified atmosphere containing 5% CO₂ at 37°C. Cells were treated with 100 µM or 500 µM H₂O₂ except as otherwise indicated elsewhere. For TNF-α-stimulated cells, cells were exposed to 10ng ml⁻¹ human TNF-α (Sigma, St. Louis, MO, USA) for 24h. To inhibit apoptosis, cells were treated with 20 µM apoptosis inhibitor Z-VAD-fmk (Promega, Madison, WI, USA). For the inhibition of necrosis, cells were treated with 30 µM necrosis inhibitor Nec-1 (Sigma).

Cell Death Assays
Cell death was measured by Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit (Invitrogen, Carlsbad, CA, USA) and was performed on a FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA). All PI positive cells (PI+) were indicated as necrotic cells, while Annexin V positive/PI negative cells (Annexin V+/PI-) were considered as apoptotic cells. In addition, TUNEL assay was performed using In Situ Cell Death Detection Kit (Roche, Hamburg, Germany) according to the manufacturer’s instruction. Necrotic cell death was assessed by propidium iodide (PI). Samples were mounted with mounting medium containing 4’,6’-diamidino-2-phenylindole (DAPI; Vector Laboratories) to stain nuclei and examined with a Zeiss LSM510 META microscope. The percentage of apoptotic nuclei was calculated by counting the total number of TUNEL-stained nuclei divided by total DAPI-positive nuclei. The percentage of necrotic cell death was calculated by counting the total number of PI-stained nuclei divided by total DAPI-positive nuclei.

Lactate dehydrogenas (LDH) release and cardiac troponin-T (cTnT) release
Necrotic cell death was measured by assessing supernatant LDH activity\(^1\). A spectrophotometric kit (Nanjing jiancheng, Jiangsu, China) was used according to the manufacturer’s instruction. In brief, 20\(\mu l\) supernatants were collected in a 96-well, then 25\(\mu l\) matrix buffer and 5\(\mu l\) coenzyme I were added to the 96-well. The mixture were incubated at 37\(^\circ\)C for 15 min. After 25\(\mu l\) 2,4-dinitrophenylhydrazine was added to each well and incubated at 37\(^\circ\)C for 15 min, 250\(\mu l\) 0.4M NaOH was added to each well and the mixture was incubated at room temperature for 5 min. The absorbance value was measured at 450nm with the spectrophotometer (BioTek, VT, USA) and the LDH activity was calculated. Plasma cTnT levels as an indicator of cardiomyocyte damage was measured using a mouse cTnT ELISA kit (Elabscience Biotechnology Co., Ltd, Wuhan, Hubei, China) according to the manufacturer’s instruction.

**RNA interference (RNAi)**

The rat FADD RNAi target sequence was 5’-CTGTGTCTTTTCTCAGAAAC-3’. A nonrelated, scrambled RNAi without any other match in the rat genomic sequence was used as a control (5’-TCAGTCTTTCTCAGAAAC-3’). The rat RIPK1 RNAi target sequence was 5’-TGAGGCTTACAACAGAGAG-3’ and the scramble sequence was 5’-CGTACTAGAGTAGGACAGAG-3’. The rat RIPK3 RNAi target sequence was 5’-GGCTTCTAAAGCAAGTGAT-3’ and the scramble sequence was 5’-GAGATATCTAGCGGATTAC-3’. The adenoviruses harboring these RNAi constructs were generated using the pSilencer adeno 1.0-CMV System (Ambion, Grand Island, NY, USA) according to the Kit’s instructions. Adenoviruses were amplified in HEK-293 cells.

Small interfering RNA (siRNA) oligonucleotides specific for TNFR1, TRADD, H19 were designed using the Ambion’s siRNA design tool, and purchased from GenePharma Co. Ltd (Shanghai, China). The siRNA sequences used were: TNFR1 siRNA, 5’-GGAUUUCUUCUAAUGCGGAAAdTdT-3’; scramble TNFR1 siRNA (TNFR1-sc), 5’-GGAUUUCACGCGUUGAAdTdT-3’; TRADD siRNA, 5’-GAAGAGCGCTTGGTTGAAATdTdT-3’; scramble TRADD siRNA (TRADD-sc), 5’-GTCAGATGAGTGAATTTdTdT-3’; H19 siRNA,
5′-GCAAGUGAUAGGAGGCCUUdTdT-3′; scramble H19 siRNA (H19-sc),
5′-GAGUACGAUCCGAGUUGAGdTdT-3′. The specificity of the oligonucleotides was
confirmed through comparing with all other sequences in Genbank using Nucleotide BLAST.
Transfection of siRNAs was performed using Lipofectamine™ 2000 (Invitrogen, Grand
Island, NY, USA) according to the manufacturer’s instructions.

**Cell transfection with miRNA duplexes or antagomirs**
The miR-103-3p and miR-107-3p duplexes were synthesized by GenePharma Co. Ltd.
MiR-103-3p mimic sequence was 5′-AGCAGCAUUGUAAGGCUAUGA-3′.
MiR-107-3p mimic sequence was 5′-AGCAGCAUUGUACAGGGCUAUA-3′. Mimic
control sequence was 5′-UUCUCCGAACGUGUCACGUTT-3′. Chemically modified
antisense oligonucleotides (antagomirs) were used to inhibit endogenous miR-103-3p and
miR-107-3p expression. The antagomir could silence both miRNAs as described. The
antagomir sequence was 5′-UCAUAGCCCUGUACAAUGCUGCU-3′. The antagomir
control sequence was 5′-CAGUACUUUUGUGUAGUACAA-3′. All the bases were
2′-O-methyl-modified (GenePharma Co. Ltd). Cells were transfected with miRNA duplexes
(100 nM) or antagomirs (100 nM) using Lipofectamine 2000 (Invitrogen) according to the
manufacturer’s instructions.

**Quantitative real-time PCR (qRT-PCR)**
Stem-loop qRT-PCR was carried out on a CFX96™ Real-time System instrument (Bio-Rad,
Hercules, CA, USA). Total RNA was extracted using Trizol reagent (Invitrogen). After DNase
I (Takara, Otsu, Japan) treatment, RNA was reverse-transcribed with reverse transcriptase kit
(Takara). The miR-103 RT primer was
5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCATAG-3′. The
miR-107 RT primer was
5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCATAG-3′.
Mature miR-103-3p and miR-107-3p levels were measured using SYBR Green Realtime PCR
Master Mix (Takala) according to the manufacturer’s instructions. The sequences of
miR-103-3p primers were: forward, 5′-CGAGCAGCAUUGUACAGGGCTATGA-3′; reverse,
5'-GTGCAGGGGTCCGAGGT-3'. The sequences of miR-107-3p primers were: forward, 5'-CGAGCAUUGUACAGGGCTATCA-3'; reverse, 5'-GTGCAGGGGTCCGAGGT-3'. The levels of miR-103-3p and miR-107-3p analyzed by qRT-PCR were normalized to that of U6. The sequences of U6 primers were: forward, 5'-GCTTCGGCAGCACATATACTAA-3'; reverse, 5'-AACGCTTCACCAGTTTGCAGT-3'. Quantitative detection of FADD, TNFR1, H19, NEAT1, KHPS1A, SNHG6, SRA1, YLPM1, CWC15, TNF-α and IL1β was also performed by qRT-PCR. The primers used for rat FADD were: forward, 5'-GGTGGCATTGGACTGTGTG-3'; reverse, 5'-TCTCCCTTACCCGATCCTGCTA-3'. The primers used for rat H19 were: forward, 5'-TAAAGCAGCTGGGTTGAGAAG-3'; reverse, 5'-TGACTGGCAGGACACATCCC-3'. The primers used for mouse H19 were: forward, 5'-GAAGAGCTCGGACTGGAGAC-3'; reverse, 5'-CTGGAGACCTGGGCTATAGGG-3'. The primers used for rat TNFR1 were: forward, 5'-GGCCAGAAACCTCAGCGAG-3'; reverse, 5'-TGCAGGCTCACTCAAGGTAGCG-3'. The primers used for rat NEAT1 were: forward, 5'-ATGGGAGGGGTAGTCAGAAG-3'; reverse, 5'-GGCTGGTGGTGGTCTAAGG-3'. The primers used for rat KHPS1A were: forward, 5'-GGACAGCTAGCTGGAGAC-3'; reverse, 5'-TGCTCTGGCTCCCTCCACTAC-3'. The primers used for rat SNHG6 were: forward, 5'-GGGAGCTGTGAGCTGGAGAC-3'; reverse, 5'-GTCGATGCTCAACCACACTGAG-3'. The primers used for rat SRA1 were: forward, 5'-AAGAAGAGGATGGCAGTCC-3'; reverse, 5'-TACTCCACCACATCCACTG-3'. The primers used for rat YLPM1 were: forward, 5'-ACCCATGGTGGAGAGAAC-3'; reverse, 5'-TGGGTGGCTCCAGAATGTCAG-3'. The primers used for rat CWC15 were: forward, 5'-GGAGAGAAATGTCAGAAG-3'; reverse, 5'-ACATCATCAGGGCCTCTCCCT-3'. The primers used for mouse TNF-α were: forward, 5'-AAACCAAGACGTGGAGGACG-3'; reverse, 5'-ACAAGGTCAAACCATCAGCGG-3'. The primers used for mouse IL1β were: forward, 5'-CGAGTTCCAGTCCAGGAG-3'; reverse, 5'-CATACAGGCTGCTAGTGC-3'. The mRNAs levels were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequences of rat GAPDH primers were: forward, 5'-TGAGTGCTTACTGGCGGTT-3'; reverse, 5'-TGTAATTCTCGGTGGTTC-3'. The sequences of mouse GAPDH primers were: forward, 5'-TTGAGTTCCGCTGGGATCTGA-3'; reverse,
5'-CCTGCTTCACCACCTTCTTGA-3'.

Adenoviral constructs

FADD and H19 adenoviral constructs were prepared using the Adeno-X™ Expression System (Clontech, Mountain View, CA, USA) according to the manufacturer’s instructions. In brief, rat H19 sequence was amplified by RT-PCR from total RNA of rat heart and then cloned into Adeno-X vector. Rat FADD cDNA was purchased from OriGene (Rockville, MD, USA). The FADD cDNA was amplified and then cloned into Adeno-X vector. The adenovirus containing β-galactosidase (β-gal) is as we described. All constructs were amplified in HEK293 cells. Adenoviral infection of cardiomyocytes was performed as we described.

Constructs of FADD-GFP reporter

The FADD cDNA was amplified and then cloned into pCMV-AC-GFP vector. The introduction of mutations in the putative miR-103/107 binding site (BS) was performed with the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) using the wild-type vector as a template. The wild-type FADD BS (FADD-Wt-GFP) sequence was TGCTGCT, the FADD BS-Mut (FADD-Mut-GFP) sequence was TCTTATT. H9c2 cells were then co-transfected with mimics (miR-103 or miR-107 or mimic control) and FADD (GFP-vector or FADD-Wt-GFP or FADD-Mut-GFP) as indicated. Cells were harvested at 24 h after transfection and the expression of GFP was monitored by the fluorescence intensity and immunoblotting.

Reporter constructs and luciferase assay

The fragment of FADD cDNA containing the miR-103/107 binding site (FADD-BS-Wt) was synthesized. The synthesized oligonucleotides were: sense, 5’-AATTCCTGGACCTGTTCTCGGTGCTGCTA-3’; antisense, 5’-CTAGTAGCAGCACCAGAAGGCTCCAGG-3’. The sense and antisense oligonucleotides were annealed and the annealed oligonucleotides were cloned into PGL3 vector (Promega, Madison, WI, USA) immediately downstream of the stop codon of the
luciferase gene. The mutant of FADD BS (FADD-BS-Mut) was cloned as the same. The synthesized oligonucleotides for FADD-BS-Mut were: sense,
5’-AATTCCTGGACCTGTTCTCGGTCTTATT-3’; anti-sense,
5’-CTAGTAATAAGACCGAGAACAGGTCCAGG-3’. For luciferase assay performed in HEK-293, cells in 24-well plates were co-transfected with 200 ng/well luciferase reporter constructs, 400 ng/well miR-103 mimic or miR-107 mimic or mimic control using Lipofectamine 2000 (Invitrogen). 5 ng/well SV-Renilla luciferase plasmids served as the internal control. Cells were harvested at 24 h after transfection and the luciferase activity was detected using the Dual Luciferase Reporter Assay kit (Promega) according to the manufacturer’s instructions. 30µl protein samples were analyzed in a luminometer. Firefly luciferase activities were normalized to Renilla luciferase activity.

**Immunoblotting**

Cells were lysed for 30 min on ice in RIPA lysis buffer (Solarbio, Beijing, China) containing 0.1 mM PMSF and a protease inhibitor (Roche). Samples were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed using the primary antibodies including anti-FADD (Abcam, Cambridge, UK), anti-RIPK1 (Abcam), anti-RIPK3 (Abcam), anti-TNFR1 (Earthox LLC., San Francisco, CA, USA), anti-Cav-1 (Santa Cruz Biotechnology), anti-TRADD (Santa Cruz Biotechnology), anti-GFP (Earthox LLC.) and anti-β-actin (Santa Cruz Biotechnology) were used. After four washes with PBS-Tween 20, horseradish peroxidase-conjugated secondary antibodies were added. The signals were detected with Pierce® ECL western blotting substrate (Pierce, Rockford, IL, USA) according to the directions of the manufacturer and exposed on x-ray films (Kodak, Rochester, NY, USA).

**Preparation of Subcellular Fractions**

Cell extracts were prepared as previously described. In brief, H9c2 cells were treated with H_{2}O_{2} for 24 hours. The cells were washed twice with PBS and harvested by centrifugation at 500g for 5 min. The pellets were resuspended in 200µl buffer A (200mM Hepes, PH 7.5, 10mM KCl, 1.5mM MgCl_{2}, 1mM EGTA, 1mM DTT, 0.1mM PMSF, 250mM sucrose)
containing a protease inhibitor (Roche). The cells were homogenized by 20 strokes in a Dounce homogenizer and centrifuged at 750g for 10 min at 4°C. The supernatants were centrifuged again at 10 000g for 10 min to collect mitochondria-enriched HM fractions and the resulting supernatants were saved as cytosolic fractions.

**Immunoprecipitation**

Cells were lysed on ice for 30 min in 500 μl NP-40 lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 10% glycerol, 0.2m M PMSF and Protease inhibitor). The lysates were precleared by centrifugation, and 50 μl of the samples were aliquoted for input. The remaining samples were immunoprecipitated with 2 μg of antibody and 50 μl of Protein-A/G PLUS-Agarose (Santa Cruz Biotechnology). The samples were rotated at 4 °C overnight. The beads were washed three times with 1 ml of low-salt NP40 lysis buffer (300 mM NaCl) and twice with 1 ml of high-salt lysis buffer (500 mM NaCl). The beads were then boiled for 10 min in the presence of 25 μl 2× sample buffer and the released proteins were fractionated in 12% SDS-PAGE gels. Proteins were detected by immunoblotting as described above.

**Pull-down assay with biotinylated miRNA**

Pull-down assay was performed as we described. Briefly, we synthesized miR-103-3p, miR-107-3p, miR-103-3p-Mut, miR-107-3p-Mut single strand RNAs which were 5′-biotin-labeled. The miR-103-3p-Mut sequence was 5′-CAAUAACUGUACAGGGCUAUAGA-3′. The miR-107-3p-Mut sequence was 5′-CAAUAACUGUACAGGGCUAUCA-3′. H9c2 cells were transfected with biotinylated miRNAs and harvested 24h after transfection. The cells were washed with PBS followed by brief vortex, and incubated in a lysis buffer (20 mM Tris-HCL, pH 7.5, 200 mM NaCl, 2.5 mM MgCl₂, 60 U mL⁻¹ RNase inhibitor, 1 mM DTT, protease inhibitor) on ice for 30 min. The lysates were precleared by centrifugation, and 50μl of the samples were aliquoted for input. The remaining lysates were incubated with streptavidin agrose beads (Invitrogen). To prevent non-specific binding of RNA and protein complexes, the beads were coated with 1% RNase-free BSA (Sigma) and 0.5mg mL⁻¹ yeast tRNA (Sigma). The beads were incubated at
4 °C for 3h, washed twice with ice-cold lysis buffer, three times with low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH8.0, 150 mM NaCl), and once with high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 500 mM NaCl). The bound RNAs were purified using Trizol for the analysis. The levels of FADD, H19, NEAT1, KHPS1A, SNHG6, SRA1, YLPM1 and CWC15 in the RNA samples were detected by qRT-PCR. The primer sequences for qRT-PCR detecting were described as above.

In vitro transcription and pull down with biotinylated H19 probe

The biotinylated RNA probe of the H19 was in vitro transcribed by mpliScribe™ T7-Flash™ Biotin-RNA Transcription Kit (Epicentre, Madison, WI, USA) according to the manufacturer’s instructions. In brief, the rat H19 was ligated to the vector PCR3.1 which contains a phage T7 transcription promoter. The plasmid was digested by NdeI and produced linear double-stranded DNA with T7 transcription promoter and 5’-protruding ends. The reaction components were combined and incubated at 37 °C for 3h. Then 1μl of RNase-Free DNase I was added to the standard 20μl reaction and incubated at 37°C for 15min in order to remove the DNA template. The transcribed RNA probes were recovered by phenol/chloroform extraction and ethanol precipitate. The probes were incubated with streptavidin agrose beads (Invitrogen) at 4 °C for 2 h to generate probe-coated beads. Cardiomyocyte lysates were incubated with probe-coated beads, and after washing with the wash/binding buffer, the RNA complexes bound to the beads were purified by Trizol and the levels of miR-103 and miR-107 were tested by RT-PCR.

Delivery of miR-103/107 antagomir, mouse model of myocardial ischemia-reperfusion (I/R) and determination of infarct sizes

Chemically modified and cholesterol-conjugated miR-103/107 antagomirs were used to inhibit endogenous miR-103/107 expression in mouse. In vivo delivery of miR-103/107 antagomir was performed as described previously. The antagomir sequence was 5’-UCAUAGCCCUGUACAAUGCUCU-3’. The antagomir negative control sequence was 5’-CAGUACUUUUGUGUAGUACAA-3’. The molecules contained 3’ cholesterol attached.
via a hydroxyprolinol linker and all the bases were 2’-O-methyl-modified (GenePharma Co. Ltd). We intravenously injected the mice on three consecutive days with antagonimir or antagonimir negative control at a dose of 20 mg kg⁻¹ body weight in 0.2 ml per injection. I/R was performed 24 hr after the last injection.

All animal experiments were performed according to the protocols approved by the Institute Animal Care Committee. C57BL/6 mice were obtained from Institute of Laboratory Animal Science of Chinese Academy of Medical Sciences (Beijing, China). For I/R experiment, 10-12-week-old male mice were anesthetized by intraperitoneal injection of a mixture of ketamine (100 mg kg⁻¹) and xylazine (5 mg kg⁻¹). We performed tracheotomy to provide artificial ventilation (0.2 ml tidal volume, 110 breaths min⁻¹) with rodent ventilator supplemented with 100% oxygen. We exposed the heart by a small left thoracotomy, identified and occluded the left anterior descending coronary artery (LAD) with 8-0 silk suture after removing the pericardium. We subjected mice to 45 min of LAD ligation followed by 1 hour of reperfusion. We explored the same procedure without ligating the suture on sham-operated mice.

To determine infarct sizes, we reoccluded the LAD and injected Evans blue (1 ml of a 2.0% solution; Sigma-Aldrich) to the ventricular cavity to demarcate the nonischemic myocardium 24 hours after ischemia-reperfusion injury. The heart was rapidly excised and sliced into 5 slices. The heart slices were incubated in 1.0% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich) for 15 minutes at 37°C for demarcation of the viable and nonviable myocardium within area at risk (AAR). The staining was stopped by ice-cold sterile saline and the slices were fixed in 10% neutral buffered formaldehyde and individually weighed. Both sides of each slice were photographed. Each of the myocardial slices was weighed and the areas of infarction (INF), area at risk (AAR), and nonischemic left ventricle (LV) were assessed with computer-assisted planimetry (NIH Image 1.57) by an observer blinded to sample identity. The ratio of AAR/LV, INF/AAR and INF/LV were calculated. AAR in the center of the territory of the left anterior descending coronary artery and the remote area in the posterior part of the left ventricle far from the AAR were prepared as described.⁷

PI and TUNEL staining of heart tissue
Necrotic cells in heart tissue after I/R injury were detected as described. In briefly, PI (10 mg kg\(^{-1}\); Sigma) was injected into the mice to label necrotic cells 1h before I/R termination. We subjected mice to 45 min of LAD ligation followed by 1 hour of reperfusion. After I/R termination, the heart was rapidly explanted, snap-frozen in liquid nitrogen, and sectioned at 6 μm thickness. TUNEL staining was performed as manufacturer’s instructions (Roche). Cardiomyocytes were identified by α-actinin and the total nuclei were stained by DAPI. Magnification ×400 photos were taken using a laser scanning confocal microscope (Zeiss LSM 510 META) and 25 random fields were quantified by an investigator who was blind to the treatment.

**Echocardiographic measurement and Masson trichrome staining**

We conducted echocardiograph at one week after ischemia-reperfusion injury using a Vevo 770 high-resolution system (Visualsonics) equipped with a 40-MHz RMV 704 scanhead and obtained parameters including diastolic left ventricular internal diameters (LVIDd) and systolic left ventricular internal diameters (LVIDs). We calculated fractional shortening (FS) as \((\text{LVIDd} - \text{LVIDs})/\text{LVIDd}\). All of the measurements were made from more than three beats. After in vivo evaluation of cardiac function the mice were euthanized and the hearts were harvested, used for Masson trichrome staining. The heart sections were stained with standard Masson trichrome staining (Sigma, St. Louis, MO) according to the manufacturer’s instructions in order to determine myocardial fibrosis resulted from I/R injury.

**Transmission electron microscopy**

Conventional electron microscopy was performed as described previously. In brief, cells were fixed with 2.5% glutaraldehyde and then postfixed with 1% osmium tetraoxide, dehydrated in agraded series of ethanol concentrations, and embedded in Embed812 resin. The ultrathin sections were mounted on copper grids and then double-stained with uranyl acetate and lead citrate. The samples were examined and photographed with a FEI Tecnai spirit transmission electron microscope.

**Statistical analysis**
All statistical analyses were performed using the SPSS 13.0 statistical software package. The results are expressed as means ± SD of at least three independent experiments. The differences among experimental groups were evaluated by one-way ANOVA analysis of variance. $p < 0.05$ was considered statistically significant.
Supporting Information References:


Online Figure I. H₂O₂ induces apoptosis and necrotic cell death in cardiomyocytes. A. Apoptotic and necrotic cells were analyzed by flow cytometry using Annexin V/PI assay in H9c2 cells treated with H₂O₂ at the indicated concentration for 24 hours. Necrosis, PI+; Apoptosis, Annexin V+/PI-. Data are means ± SD (n=4). *p<0.05 versus control. B. LDH activity was measured. H9c2 cells were exposed to 100 μM H₂O₂ or 500 μM H₂O₂ at the indicated time. Data are means ± SD (n=3). C. Electron microscopy (EM). Cells were treated with 100 μM H₂O₂ or 500 μM H₂O₂ for 24 hours. Representative images showed the characteristics of apoptotic and necrotic cells (n=3). Scale bar, 5μm. D. Necrotic cells were analyzed using PI assay in H9c2 cells exposed to 30 μM Nec-1 and/or 500 μM H₂O₂ for 24 hours or 12 hours. Data are means ± SD (n=4). *p<0.05 versus H₂O₂ alone. E-H. Necrotic and apoptotic cells were analyzed using Annexin V/PI assay. Necrosis, PI+; Apoptosis, Annexin V+/PI-. (E-F) H9c2 cells were transfected with FADD-siRNA or its scramble form (FADD-sc) and exposed to 100 μM H₂O₂ for 12 hours (E) or 24 hours (F). (G-H) H9c2 cells were infected with FADD or β-gal and exposed to 500 μM H₂O₂ for 12 hours (G) or 24 hours (H). Data are means ± SD (n=3). *p<0.05 versus H₂O₂ alone. I-J. LDH activity was measured in H9c2 cells treated as described in E (I) or G (J). Data are means ± SD (n=3). *p<0.05 versus H₂O₂ alone.
Online figure II

A
RIPK1
RIPK3
Actin
0 6 12 24 (h)
H₂O₂ (100 µM)

B
RIPK1
RIPK3
Actin
0 6 12 24 (h)
H₂O₂ (500 µM)

C
RIPK1
Actin
RIPK1-siRNA
RIPK1-sc

D
RIPK3
Actin
RIPK3-siRNA
RIPK3-sc

E
IP: Anti-RIPK1

F
IP: Anti-RIPK1

G
Cytosol
IP: Anti-RIPK1

H

I

H₂O₂ (100 µM)
RIPK1-sc
RIPK1-siRNA

Annexin V + PI-
cells (%)
Online Figure II. FADD regulates necrosis through influencing the complex of RIPK1/ RIPK3. A-B. RIPK1 and RIPK3 protein levels were detected by immunoblot in H9c2 cells treated with 100 μM H2O2 (A) or 500 μM H2O2 (B) at the indicated time. C-D. RIPK1 and RIPK3 protein levels were analyzed by immunoblot in H9c2 cells infected with adenoviral RIPK1-siRNA (C) or RIPK3-siRNA (D). E. H2O2-induced interaction of RIPK1 with RIPK3 was inhibited by adding Nec-1. H9c2 cells were pretreated with 30 μM Nec-1 for 24 hours and then exposed to 500 μM H2O2 for 6 hours. The interaction of RIPK1 with RIPK3 was detected by immunoprecipitation. F. Knockdown of TRADD did not increase the interaction of RIPK1 with RIPK3. The interaction was detected by performing the immunoprecipitation in H9c2 cells transfected with TRADD-siRNA or TRADD-sc and exposed to 100 μM H2O2 for 6 hours. G. RIPK1 was interacted with RIPK3 both in cytosol fraction and heterotypic membrane (HM) fraction. H9c2 cells were treated with 500 μM H2O2 for 6 hours, then cytosol fraction and HM fraction were prepared as described in Supplemental material. Tublin served as cytosol marker. Cox IV served as HM marker. H-I. Knockdown of RIPK1 or RIPK3 prevented H2O2-induced apoptosis. Apoptosis was analyzed using Annexin V/PI assay in H9c2 cells infected with RIPK1-siRNA (H) or RIPK3-siRNA (I) and exposed to 100 μM H2O2 for 24 hours. Apoptosis, Annexin V+/PI-. Data are means ± SD (n=3). *p<0.05 versus H2O2 alone.
Online Figure III. MiR-103/107 participate in the regulation of FADD expression. A. The wild type FADD CDS containing the binding site of miR-103/107 (FADD-BS-Wt) (up) or its mutant (FADD-BS-Mut) (down) were cloned into pGL3 as indicated. The mutant sites were labelled in red. B. Constructs of FADD wild-type-GFP (FADD-GFP, up) and a mutated FADD-GFP in the miR-103/107-binding sites (FADD-Mut-GFP, down). The mutant sites were labelled in red. C. The association of FADD mRNA and miR-103/107 was detected by biotin-based pulldown assay. H9c2 cells were transfected with biotinylated miR-103 (Bio-103) or biotinylated miR-107 (Bio-107) or negative control (Bio-NC) to perform biotin-based pull down. FADD levels were analyzed by RT-PCR and run on 2% agarose gel.
Online Figure IV

Necrosis was not changed in H9c2 cells transfected with Anta-103/107 or Anta-NC and exposed to 100 μM H2O2 for 24 hours. Necrosis was measured using PI assay. Data are means ± SD (n=3). B. Necrosis was not changed in H9c2 cells transfected with miR-103/107 or NC at the indicated time. Necrosis was measured using PI assay. Data are means ± SD (n=3). C. MiR-103/107 increased H2O2-induced necrosis. Necrosis was measured using PI assay in H9c2 cells transfected with miR-103 or miR-107 or NC and treated with 500 μM H2O2 for 24 hours. Data are means ± SD (n=3). *p<0.05 versus H2O2 alone. D. Cav-1 protein levels were detected by immunoblot in H9c2 cells treated with 500 μM H2O2 at the indicated time. E. Knockdown of miR-103/107 abolished the decrease of cav-1 induced by H2O2. H9c2 cells were transfected with Anta-103/107 or Anta-NC and exposed to 500 μM H2O2 for 24 hours. The protein levels of cav-1 were measured by immunoblot.
Online Figure V. MiR-103/107 are not involved in TNF-α induced necrosis. The TNFR1 expression levels were detected by immunoblot in H9c2 cells transfected with TNFR1-siRNA or its scramble form (TNFR1-sc). The blots showed the representative protein levels of TNFR1.
Online figure VI

A) miRNA levels (fold change)

B) miRNA levels (fold change)

C) TnI (μg/L)

D) TUNEL, DAPI, Actinin, Overlay

E) Collagen area (%)
Online Figure VI. MiR-103/107 regulate necrotic cell death in heart. A. MiR-103 and miR-107 levels were analysed by qRT-PCR in mice injected with Anta-103/107 or Anta-NC. Data are means ± SD (n=6 mice per group). *p<0.05 versus control. B. The expression levels of miR-103 and miR-107 were analyzed by qRT-PCR and the expression of FADD was analyzed by immunoblot. Mice were treated as described in Figure 6D. Data are means ± SD (n=8 mice per group). *p<0.05 versus I/R alone. C. Cardiac troponin T (cTnT) was measured in mice treated as described in Figure 6D. Plasma cTnT was measured as described Supplementary Methods. Data are means ± SD (n=6 mice per group). *p<0.05 versus I/R alone. D. Apoptosis were detected by TUNEL assay in mice treated as described in Figure 6D. Representative images of ventricular myocardium sections from sham operation or I/R (up) and the quantitative analysis of necrosis is shown (down). Green, TUNEL-positive nuclei; blue, DAPI-stained nuclei; red, cardiomyocytes labeled with antibody to α-actinin; scale bar, 50 µm. Data are means ± SD (n=6 mice per group). E. Collagen deposition of the peri-infarct area in the heart section was analyzed by Masson’s trichrome staining. The quantitative analysis of collagen area is shown. Data are means ± SD (n=8 mice per group). *p<0.05 versus I/R alone.
Online figure VII

A

B

miR-103

3' - AGUAUCGGGACAUcGU - UACGAGCA-5'

5' - AAAGAGCUAC-AACUCUGCGCUGCU -

Position 1540-1564

3' - AGUAUCGGG-ACA-UGUUAUCACAGA-5'

5' - UGUAAGCGCCCUUGUUGCUGCAGCU -

Position 2107-2132

Rat H19

5' - BS1 BS2 BS3 - 3'

miR-107

3' - ACUAUCGGGACAUcGU - UACGAGCA-5'

5' - AAAGAGCUAC-AACUCUGCGCUGCU -

Position 1540-1564

3' - ACUAUCGGG-ACA-UGUUAUCACAGA-5'

5' - UGUAAGCGCCCUUGUUGCUGCAGCU -

Position 2107-2132

Rat H19

5' - BS1 BS2 BS3 - 3'

C

D

E

H19 levels (fold change)

H19 levels (fold change)

H19 levels (fold change)

0 0.4 0.8 1.2

60 5 10 30 60

Sham Ischemia (min)

0 0.4 0.8 1.2

1 2 4 6

β-gal - -

H19 - - +

H19-siRNA - - +

*
Online Figure VII. H19 interacts with miR-103/107 and regulates miR-103/107 expression. A. The expression levels of LncRNA H19, NEAT1, KHPS1A, SNHG6, SRA1, YLPM1 and CWC15 were analyzed by qRT-PCR in H9c2 cells exposed to 100 μM H2O2 or 500 μM H2O2 for 24 hours. Data are means ± SD (n=3). *p<0.05 versus control. B. Putative miR-103 and miR-107 binding sites in H19. This putative miR-103 and miR-107 binding sites in H19 were analyzed by RNA hybrid program. C. The levels of H19 were detected in mice subjected to ischemia at the indicated time. Data are means ± SD (n=8 mice per group). *p<0.05 versus sham. D-E. The expression levels of H19 were detected in H9c2 cells infected with H19 (D) or H19-siRNA (E). Data are means ± SD (n=3). *p<0.05 versus control. F. The expression levels of H19 were analyzed in H9c2 cells infected with H19 or β-gal and exposed to 500 μM H2O2 for 24 hours. Data are means ± SD (n=3). *p<0.05 versus H2O2 alone. G. The sequence of miR-103-Wt, miR-103-Mut, miR-107-Wt and miR-107-Mut. The mutated sequence of miR-103-Mut and miR-107-Mut was labelled. H. LncRNA NEAT1, KHPS1A, SNHG6, SRA1, YLPM1 and CWC15 were not associated with miR-103/107. H9c2 cells were transfected with Bio-103 or Bio-107 or Bio-NC, then biotin-based pull down assay was performed. The levels of NEAT1, KHPS1A, SNHG6, SRA1, YLPM1 and CWC15 were detected by qRT-PCR and normalized to Bio-NC. Data are means ± SD (n=3).
Online Figure VIII.

**A.** The expression levels of H19 were analyzed in H9c2 cells treated with TNF-α alone or TNF-α combination with z-VAD-fmk for 24 hours. Data are means ± SD (n=3).

**B.** Necrosis was measured using PI assay in H9c2 cells transfected with H19-siRNA or H19-sc and exposed to TNF-α for 24 hours. Data are means ± SD (n=3). *p<0.05 versus TNF-α alone.