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Original Article

miR-432 exerts a protective effect against myocardial ischemia/reperfusion injury by activating the β-catenin/HIF-1α pathway and augmenting NRF2-mediated anti-oxidative stress

Rui-Ying Su[1], Wei-Yan Tai[2], Hong-Shan Yin[2], Xiao-Yong Geng[2]

[1]Department of Cardiac Function Inspection, The Third Hospital of Hebei Medical University, Shijiazhuang 050051, Hebei, China; [2]Department of Cardiology, The Third Hospital of Hebei Medical University, Shijiazhuang 050051, Hebei, China

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Abstract: Objective: MicroRNAs (miRNAs) have been shown to play an important role in myocardial ischemia/reperfusion (MI/R) injury. This study aimed to determine the role of miR-432 in MI/R injury. Methods: We established a MI/R injury model by ligation/untying of the left anterior descending coronary artery, and used viral infection to regulate gene expression, such as that of miR-432 *in vitro* and *in vivo*, and used RT-qPCR to detect the expression of the gene at mRNA level. Finally, western blotting and immunochemistry analyses were used to determine the protein level. Results: The results of this study show that miR-432 is upregulated in the heart following MI/R injury and that miR-432 overexpression showed a significant decrease, while miR-432 knockdown showed a significant increase in the ratio of the infarct area (IA) to the area at risk (AAR) and levels of serum creating phosphokinase (CPK). Moreover, miR-432 augmented the activation of the β-catenin pathway and decreased the rate of apoptosis in the mice heart at 24 hours after MI/R injury by targeting RBM5. At the same time, miR-432 overexpression enhanced HIF-1α activation, while β-catenin deletion attenuated HIF-1α activation induced by miR-432 overexpression. Importantly, β-catenin and HIF-1α knockdown significantly increased the rate of apoptosis and the ratio of IA to AAR and levels of serum CPK induced by miR-432 overexpression at 24 hours after MI/R injury. miR-432 overexpression strongly decreased levels of SOD and GSH-PX activity, and increased levels of MDA activity and the expression of the $gp91^{phox}$ protein in the mice hearts at 24 hours after MI/R injury, while miR-432 knockdown exerted an opposite effect. miR-432 was also found to have increased NRF2 protein levels by targeting KEAP1 protein expression. NRF2 knockdown reversed the downregulation of the levels of $gp91^{phox}$ protein and MDA, while it also reversed the upregulation of the levels of SOD and GSH-PX induced by miR-432 overexpression in the heart of the mice at 24 hours after MI/R injury. Conclusion: miR-432 protects against MI/R injury by activating the β-catenin/HIF-1α pathway and augmenting NRF2-mediated anti-oxidative stress.

Keywords: microRNA, myocardial ischemia/reperfusion injury, apoptosis, oxidative stress

Introduction

Myocardial ischemia (MI) is a pathological state that occurs as a result of coronary artery lumen occlusion or stenosis. The high incidence of MI makes it a huge threat to human health [1, 2]. Although myocardial reperfusion can be achieved clinically through many methods of treatment, including percutaneous coronary intervention, coronary artery bypass grafting, as well as antiplatelet and anticoagulant drugs, it may still cause deterioration of heart function and myocardial damage following myocardial reperfusion [3], which results in myocardial ischemia/reperfusion (MI/R) injury. Cardiac remodeling induced by MI/R injury is the main cause of heart failure and can increase long-term mortality and decrease the quality of life of patients with myocardial ischemia [4, 5]. Therefore, elucidation of the mechanism of action of MI/R injury is very important for decreasing the incidence of heart failure and improving the quality of life of MI patients.

miRNAs have received increased attention, not only because miRNAs play important roles in

cardiac development, cardiac remodeling, cardiac hypertrophy, cardiomyocyte apoptosis, arrhythmia, and heart failure [6, 7], but also because an increasing amount of evidence has indicated the critical involvement of miRNAs, such as miR-21 [8], miR-101 [9], miR-19a [10], and miR-26a [11], in the regulation of cardiac injury and dysfunction induced by MI/R injury. Importantly, MI/R-related miRNAs have been found to act as drug targets for MI/R injury treatment, such as gypenoside A, which attenuates MI/R injury by inhibiting the miR-143-3p expression via activating the AMPK/Foxo1 pathway [12]. miRNA-204 was found to be upregulated by sevoflurane to ameliorate MI/R injury in mice by inhibiting Cotl1 expression [13]. miR-432 is a miRNA that is often mentioned in cancer research, such as in studies on breast cancer, cervical cancer, liver cancer, and glioma. Interestingly, although there are only a few related studies, previous studies have found that miR-432 is involved in heart disease caused by cardiac hypertrophy [14] and heart failure [15]. However, the effect of miR-432 on MI/R injury is still unclear.

The Wnt/β-catenin pathway is involved in a variety of cellular activities and plays an important role in a variety of diseases, including myocardial ischemia-reperfusion [16, 17]. Its mechanisms of action include inhibiting myocardial apoptosis induced by MI/R injury [18] and augmenting the adaptive response by activating the hypoxia-inducible factor 1α (HIF-1α) signaling pathway [19]. Importantly, miR-432 has been found to activate the wnt/β-catenin pathway in human hepatocellular carcinoma [20] and bladder cancer cells [21]. Oxidative stress is also an important mechanism involved in MI/R injury, and NRF2-mediated anti-oxidative stress has been found to reduce MI/R injury induced by oxidative stress [22, 23]. Interestingly, miR-432 can induce NRF2 stabilization by directly targeting KEAP1 [24]. However, these theoretically important molecular mechanisms in MI/R injury regulated by miR-432 have not been studied in vivo. In this study, we demonstrated the protective effect exerted by miR-432 against MI/R injury in a mouse MI/R model, while its molecular mechanism might be associated with the activation of the β-catenin/HIF-1α pathway by targeting RBM5 and augmenting NRF2-mediated anti-oxidative stress by targeting KEAP1.

Materials and methods

Animal and MI/R models

All animal experiments were reviewed and approved by the local ethics committee (Number G2020-021-1). C57BL/6J male mice were (8-10 weeks old, 20-26 g, n = 112) housed in a pathogen-free environment (temperature, 23±2°C; humidity, 55±5%) under 12 h light/dark cycles and free access to food and water. The mice were used to establish a MI/R model, as previously described [19, 25]. In brief, after being anesthetized using 100 mg/kg ketamine and 10 mg/kg xylazine i.p., the left anterior descending (LAD) coronary artery was immediately identified and ligatured using a 6.0 silk suture. After 30 minutes of ischemic reperfusion, reperfusion of the LAD was performed for 12, 24, 48, 72, and 96 hours. The chest of the mice in the sham group were cut open but the LAD was not ligatured. The mice were euthanized using 100% carbon dioxide inhalation for 5 minutes. We confirmed that the mice had died by observing the heartbeat and breathing of the mice.

miR-432 expression assay

Levels of miR-432 expression in the heart tissues were detected using quantitative real-time PCR (qPCR). First, the RNAiso regent (9108, TARAKA) was used to extract total RNA from the heart tissues. Then, the PrimeScript RT reagent (RR047A, Taraka) was used to prepare the cDNA. Finally, 20 μL of the qPCR mixture was prepared following instructions provided in the progema qPCR mix kit (A6001, progema) and was analyzed using an Applied Biosystems PCR system. Cycling conditions for the PCR experiment were as follows: 95°C for 2 minutes, 40 cycles of (95°C for 15 seconds and 60°C for 1 minute). The qPCR primers used were as follows: miR-432-F: 5'-GCTCTTGGAGTAGGTCATTGGGTG-3'; miR-432-R: 5'-TGGTGTCGTGGAGTCG-3'; U6-F: 5'-CTCGCTTCGGCAGCACA-3'; U6-R: 5'-AACGCTTCACGAATTTGCGT-3'. In this study, we calculated the relative expression level of miR-432 using the $2^{-\Delta\Delta Cq}$ method [26], while U6 was used as the loading control.

Knockdown and overexpression of specific genes

Deletion of β-catenin, HIF-1α, and NRF2 in the heart tissues of mice was performed using

small interfering RNAs (siRNAs) specific for β-catenin (siβ-catenin), HIF-1α (siHIF-1α), and NRF2 (siNRF2), and the negative control siRNA (sictrl). miR-432 was knocked down in the heart tissues of the mice using anti-miR-432 (AM17000, Invitrogen) and the corresponding control sequence (Anti-ctrl) was used as the control. siβ-catenin, siHIF-1α, siNRF2, sictrl, anti-miR-432, or anti-ctrl were mixed with the in vivo-jetPEI reagent (Polyplus, 201-10G) and administered through intra-myocardial injection 48 hours prior to MI/R surgery. *In vitro*, 10 ul of AAV-miR-432 and AAV-ctrl, 50 nM anti-miR-432, and anti-ctrl were directly transfected into H9c2 cells (2.5×10^6) using a lipofectamine™ 2000 transfection reagent kit (Invitrogen, 11668019) at 37°C for 36 hours. The siRNA sequences are as follows: siβ-catenin: 5'-AAUCACAAACCUUGAGUAGCC-3', si-HIF-1α: 5'-AAAAACUUCAGACUCUUUGCU-3', si-NRF2: 5'-ACUUACUCCAAGAUCUAUGUC-3', sictrl: 5'-CUUUUUUGCUCAGUUUCAACU-3'.

For the overexpression of RBM5 and miR-432 in the heart of the mice, 30 μl of adenovirus was delivered through intra-myocardial injection 2 weeks prior to MI/R surgery was used to overexpress miR-432 (AAV-miR-432) (Vector Biolabs, AAV-MI0012528) or RBM5 (AAV-RBM5) (Vector biolabs, AAV-270268), while empty adenovirus (AAV-ctrl) was used as the control.

Measurement of the myocardial infarction area

24 hours after reperfusion, we injected 1% Evans blue into the aorta of the mice and harvested the heart tissues to prepare the heart sections. Then, 1% 2,3,5-triphenyl tetrazolium chloride (TTC) was used to stain the heart slices and images were captured under a photomicroscope. Finally, we used ImageJ software 6.0 (NIH, USA) to quantify the size of the left ventricular area (LVA), area at risk (AAR), and infarct area (IA).

Measurement of SOD, GSH-PX, and MDA activity

After 24 hours of reperfusion, the mice heart tissues were harvested to detect the activity of SOD (Solarbio, BC0170), GSH-PX (Leagene, T01033), and MDA (Abcam, ab118970).

Protein expression assay

We extract total protein from cells and tissues (Shanghai Shangbao Biotechnology Co., Ltd., 26110) and determined the protein concentration using ultraviolet spectrophotometry. Next, we analyzed the protein composition using SDS-PAGE by transferring it onto a PVDF membrane. After being sealed using 5% skimmed milk at room temperature for 1 hour, we incubated the sample with β-catenin (1:1000, Abcam, ab184919), Bax (1:1000, Abcam, ab32-503), Bcl2 (1:1000, Abcam, ab182858), cleaved caspase 3 (1:500, Abcam, ab214430), NRF2 (1:2000, Cell Signaling Technology, 127-21), RBM5 (1:1000, Cell Signaling Technology, 86425), KEAP1 (1:2000, Abcam, ab139729), and HIF-1α (1:1000, R&D, 241809) antibodies, as a primary antibodies, overnight at 4°C. After being washed by PBS buffer for three times, we incubate the corresponding secondary antibody at room temperature for 1 hour. At last, we use chemical developer (Beijing Pulilai Gene Technology Co., Ltd., P1020) to develop the protein bands obtained from the PVDF membrane, and used ImageJ software to analyze the g ray value of the protein bands.

TUNEL staining of the apoptosis cells

We used paraformaldehyde to fix the mice hearts at 4°C overnight, embed them in paraffin and sliced the tissue into sections. After dehydration and dewaxing, proteinase K was incubated into the tissue sections. As described in the instructions provided by the manufacturer of the TUNEL Cell Apoptosis Detection Kit (TRANSGEN, TA201-02), we visualized the TUNEL positive cells in the heart tissues. TUNEL positive cells were counted in 5 random fields of 3 different sections at 100 × magnification using TissueFAXS Confocal Plus 200 (Leica).

Statistical analysis

Data presented in this study were record and analyzed using SPSS 20.0 software (IBM, USA). The measurement data were expressed as (mean ± standard deviation), and the difference between the two groups were compared using the unpaired t-test and between multiple groups were compared using one-way ANOVA along with the turkey post hoc test. A P value of <0.05 was used to indicate a significant difference.

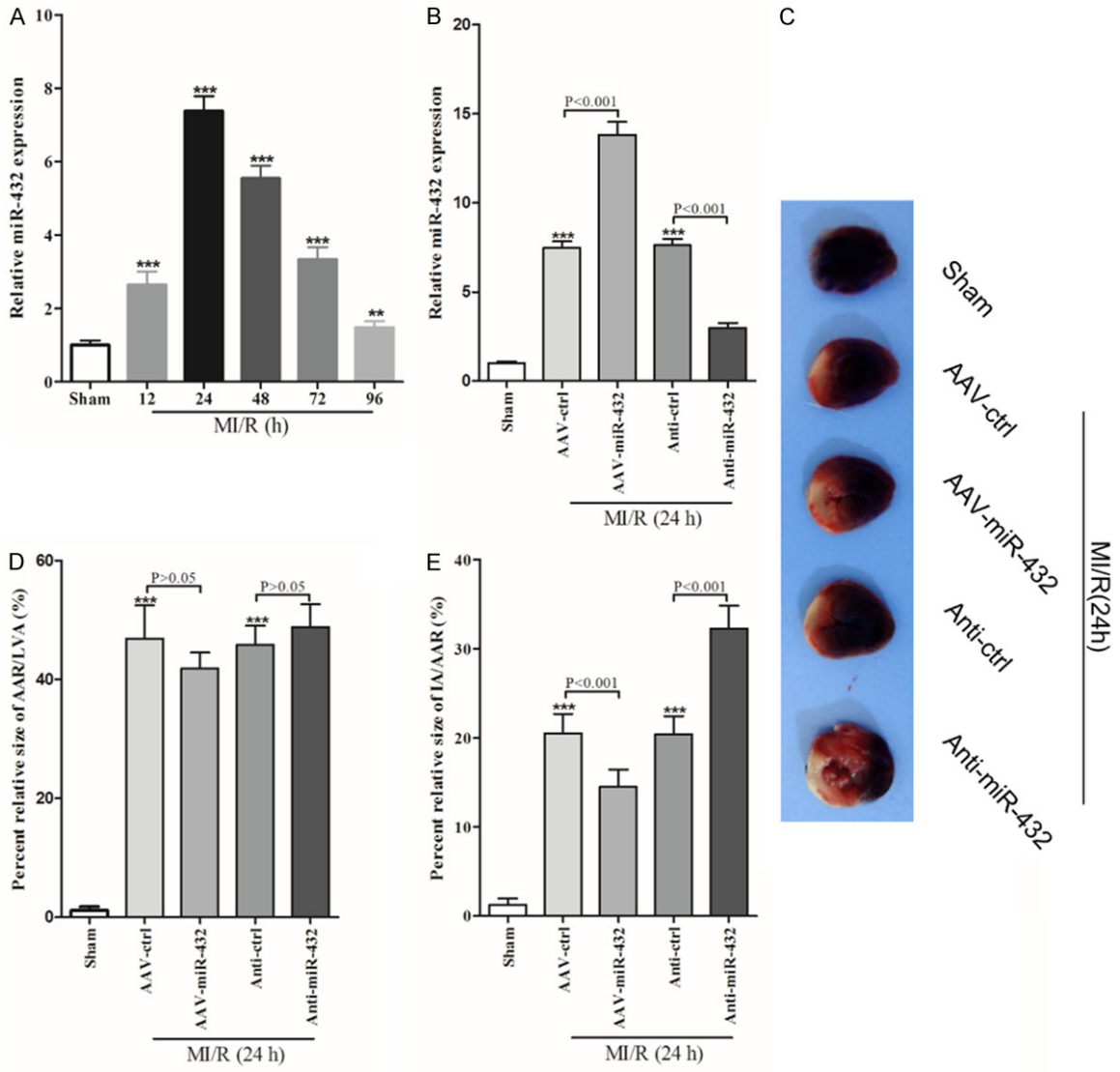

Figure 1. MI/R induces increased miR-432 expression, and miR-432 protects against MI/R injury. (A) miR-432 expression was analyzed by qPCR in heart of mice following different time (12, 24, 48, 72 and 96 hours) of MI/R injury. 8 mice each group, and *P* value was calculated by post-hoc comparisons. **P<0.01 and ***P<0.001 vs Sham group. (B) miR-432 mixed transfection reagent was injected into the myocardium of mice to regulate miR-432 expression 48 hours prior to MI/R injury, and analyzed miR-432 expression. (C) Representative cardiac Evans blue-TTC staining. (D, E) miR-432 mixed transfection reagent was injected into the myocardium of mice to regulate miR-432 expression 48 hours prior to MI/R injury, and the percent of area at risk (AAR) to left ventricular area (LVA) (D), percent of infarct area (IA) to AAR (E) at 24 hours of MI/R injury. 8 mice each group, *P* value was calculated by post-hoc comparisons, **P<0.01 and ***P<0.001 vs Sham group.

Results

miR-432 is upregulated and protects against MI/R injury

The results of the qPCR analysis indicated that miR-432 expression was rapidly upregulated during the first 24 hours of reperfusion, and reached a peak at 24 hours after MIR/R injury. Then, gradually decreased during the following 24 hours until 96 hours after reperfusion (**Figure 1A**). The increase in miR-432 expression after MI/R injury indicates that miR-432 plays a role in MI/R injury. To test this possibility, we regulated the expression of miR-432 in the heart tissues using an intramyocardial injection of miR-432 mixed with a transfection reagent, and the results of the qPCR analysis

showed that AAV-miR-432 could significantly upregulate miR-432 expression, compared with the AAV-ctrl group at 24 hours of MI/R injury, while anti-miR-432 could also significantly decrease miR-432 expression, compared with the anti-ctrl group, at 24 hours after MI/R injury (**Figure 1B**). Next, we assessed the degree of MI/R injury by determining the percentage of the area at risk (AAR) to the left ventricular area (LVA), the percentage of the infarct area (IA) to AAR, and the level of serum creatine phosphokinase. The results showed that although miR-432 overexpression or miR-432 knockdown did not significantly affect the ratio of AAR to LVA (**Figure 1C** and **1D**), miR-432 overexpression significantly decreased the IA to AAR ratio (**Figure 1C** and **1E**). Overall, these results indicate that the upregulation of miR-432 protects against MI/R injury, by decreasing the IA to AAR ratio and the level of serum creatine phosphokinase after MI/R injury.

miR-432 enhances β-catenin activation and decreases MI/R-induced apoptosis

MI/R-induced cardiomyocyte apoptosis is directly associated with MI/R injury, and the inhibition of MI/R-induced cardiomyocyte apoptosis is crucial in preventing MI/R injury [27, 28]. The activation of β-catenin is important for MI/R injury, while the activation of β-catenin also plays a central role in the regulation of apoptosis [16, 17]. Importantly, previous studies have found that the β-catenin-mediated signal pathway is a downstream pathway regulated by miR-432 [20]. However, as far as we know, it is unclear whether the protective effect exerted by miR-432 against MI/R injury is associated with β-catenin-mediated anti-apoptosis. Therefore, we detected the expression of key proteins involved in the activation of β-catenin and cardiomyocyte apoptosis after MI/R injury. We found that MI/R activated the β-catenin pathway, compared with the sham group, and that miR-432 overexpression significantly evaluated β-catenin protein expression (**Figure 2A** and **2B**) and the p-GSK-3β/GSK-3β protein expression ratio (**Figure 2A** and **2C**). At the same time, cardiomyocyte apoptosis-related proteins were also found to be significant, that is that the ratio of Bax/Bcl2 (**Figure 2D**) and cleaved-caspase 3 protein (**Figure 2E**) were all significantly increased, compared with the sham group in the heart of mice following MI/R injury, and that miR-432 overexpression led to the significant decrease in the ratio of Bax/Bcl2 and cleaved-caspase 3 protein expression, while miR-432 knockdown produced an opposite result. Importantly, we also determined the rate of apoptosis in heart sections using TUNEL staining and found that MI/R damage increased the proportion of cardiac apoptotic cells, while the decrease in miR-432 overexpression and miR-432 knockdown increased the rate of apoptosis (**Figure 2F**).

miR-432 enhances β-catenin activation by targeting RBM5

We investigated the mechanism by which miR-432 regulates β-catenin activation in MI/R injury. As is well-known, miR-432 is a non-coding RNA that regulates gene expression by binding to its target gene mRNA sequence. However, we did identify the binding site of miR-432 in the β-catenin sequence. A previous study found that miR-432 could increase β-catenin expression by targeting RBM5. Similarly, our study also found a binding site of miR-432 at the 25819-25824 position of RBM5 (**Figure 3A**). To investigate whether miR-432 could target RBM5 in cardiomyocytes, we first transfected the luciferase reporter containing WT or mut RBM5 sequences into H9c2 cells, and then regulated miR-432 expression to compare the activity of luciferase in the H9c2 cells. The results of the dual luciferase reporter experiment showed that miR-432 overexpression could significantly decrease luciferase activity in the H9c2 cells transfected with the luciferase reporter containing WT-RBM5 sequences. Similarly, miR-432 deletion could significantly increase the activity of luciferase in H9c2 cells transfected with luciferase reporter containing WT-RBM5 sequences (**Figure 3B**). At the same time, we found that miR-432 overexpression significantly decreased RBM5 protein levels in the H9c2 cells and that miR-432 deletion significantly increased RBM5 protein levels (**Figure 3C**).

To study the regulatory effect of miR-432 on RBM5-mediated β-catenin expression *in vivo*, we regulated miR-432 and RBM5 expression in the heart tissues by transfecting miR-432 mixed with the transfection reagent. As shown, AAV-RBM5 could significantly increase the expression of the RBM5 protein in the heart tis-

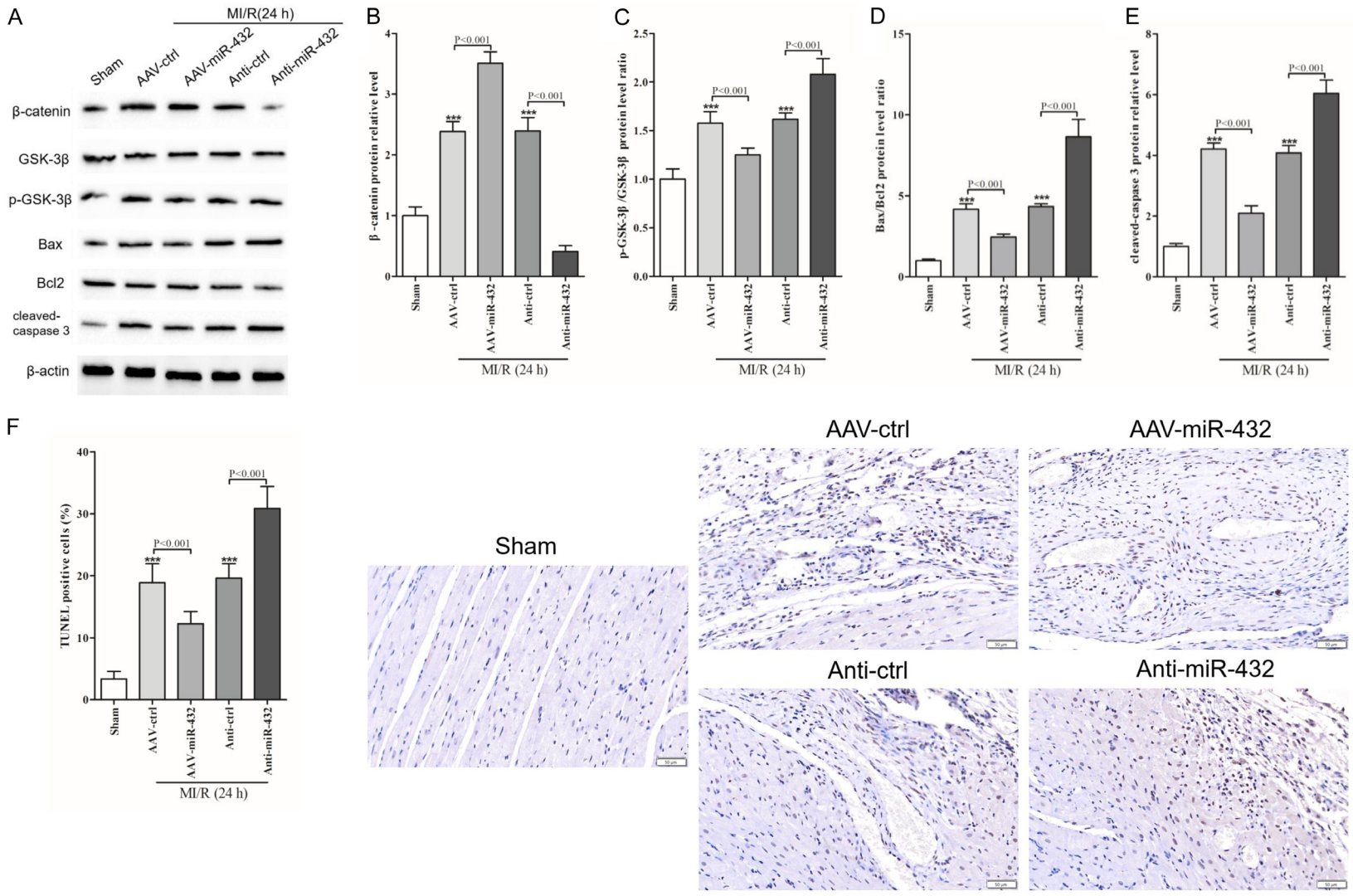

Figure 2. miR-432 augments the activation of β-catenin pathway and decreases apoptosis after MI/R injury. miR-432 mixed transfection reagent was injected into the myocardium of mice to regulate miR-432 expression 48 hours prior to MI/R injury. 24 hours of MI/R injury, we harvested the mice heart and used western blot method (A) to analyze the expression of β-catenin (B), p-GSK-3β and GSK-3β (C), Bax and Bcl2 (D), and cleaved-caspase 3 (E) protein expression, and used TUNEL staining to analyze the cardiomyocyte apoptosis (F). 8 mice each group, *P* value was calculated by post-hoc comparisons, ***P<0.001 vs Sham group.

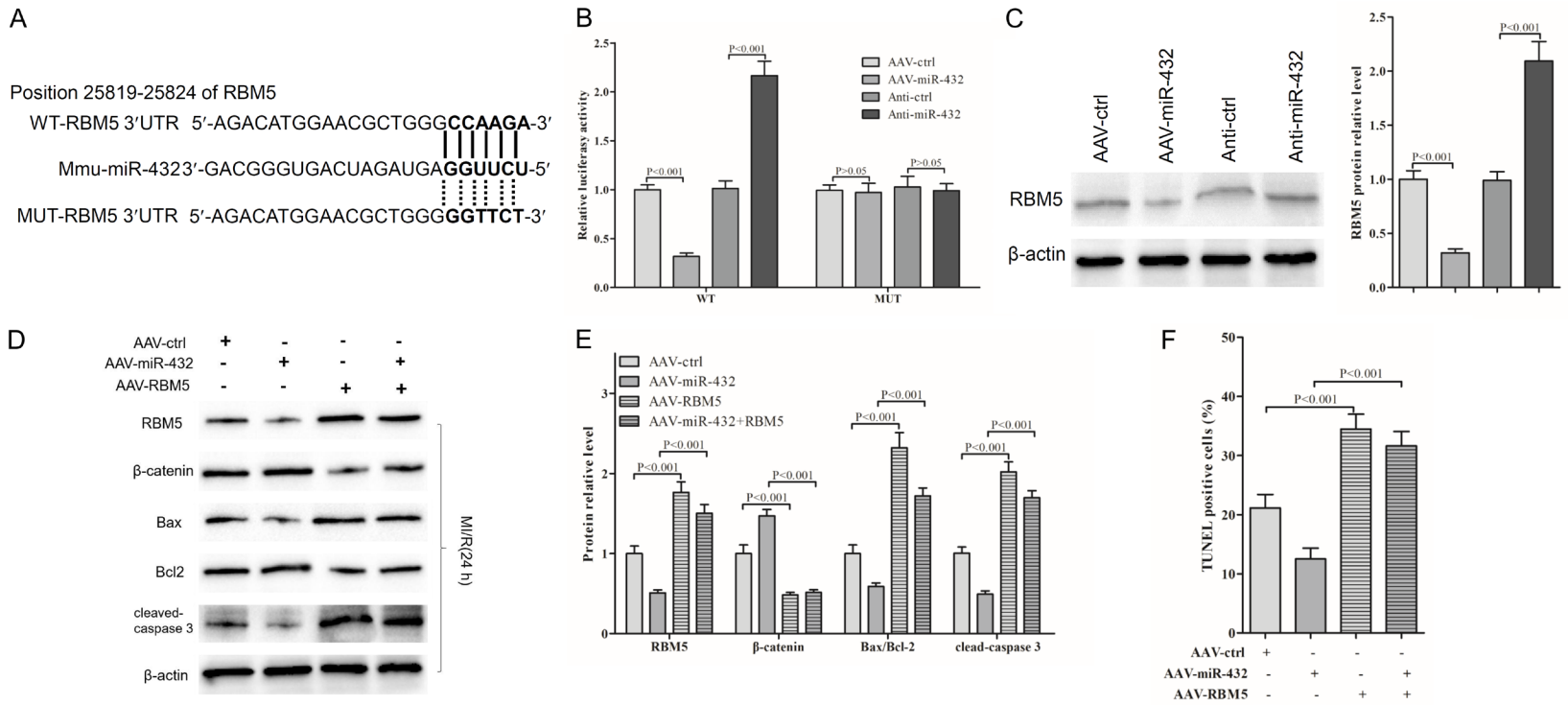

Figure 3. miR-432 targets to inhibit RBM5 expression and RBM5 overexpression attenuates miR-432 protective effect on apoptosis after MI/R injury. (A) Wild type (WT) or mutation sequence of RBM5 binding to miR-432. (B) Luciferase reporter containing WT or mut RBM5 sequences were transfected into H9c2 cells prior to transfectmiR-432 mixed transfection reagent, and harvested the activity of luciferase. 3 independent repetitions, *P* value was calculated by student's t test. (C) Western blot analysis of RBM5 protein expression at 24 hours after transfecting miR-432 mixed transfection reagent in H9c2 cells. (D, E) Mixed transfection reagent was injected into the myocardium of mice to regulate miR-432 and RBM5 expression 48 hours prior to MI/R injury, and used western blot (D) to analyze RBM5, β-catenin, Bax and Bcl2, and cleaved-caspase 3 protein expression (E). 8 mice each group, *P* value was calculated by student's t test. (F) Mice were treated as descripted in (D, E), and used TUNEL staining to analyze the cardiomyocyte apoptosis. 8 mice each group. 3 independent repetition, *P* value was calculated by student's t test.

sues at 24 hours after MI/R injury (**Figure 3D** and **3E**). Importantly, RBM5 overexpression could not only significantly decrease the expression of β-catenin protein, but could also significantly decrease β-catenin protein expression induced by miR-432 overexpression (**Figure 3D** and **3E**). Moreover, we also assessed the rate of MI/R-induced apoptosis in the heart tissues of the mice infected with miR-432 overexpression mixed with the transfection reagent or RBM5 overexpression adenovirus. The results of the western blotting analysis showed that RBM5 overexpression could not only significantly increase the ratio of Bax/Bcl2 protein expression and cleaved-caspase 3 protein expression, but also significantly increased the decrease in the ratio of Bax/Bcl2 protein expression and cleaved-caspase 3 protein expression induced by miR-432 overexpression at 24 hours after MI/R injury (**Figure 3D** and **3E**). The results of the TUNEL staining also showed that RBM5 overexpression significantly reversed the decrease in the rate of cardiomyocyte apoptosis induced by miR-432 overexpression at 24 hours after MI/R injury (**Figure 3F**).

miR-432 decreases the rate of MI/R-induced apoptosis through the β-catenin/HIF-1α pathway

We regulated miR-432 expression in the heart tissues by transfecting them with the miR-432 mixed transfection reagent, and regulated β-catenin expression by transfecting the tissues with a siRNA specific to β-catenin (siβ-catenin), while siRNA (sictrl) and siβ-catenin, which were used as the negative controls, respectively, which significantly decreased β-catenin levels and attenuated HIF-1α activation in the heart tissues at 24 hours after MI/R injury (**Figure 4A**). More importantly, β-catenin could significantly decrease elevated HIF-1α protein expression induced by miR-432 overexpression in the heart tissues of the mice at 24 hours after MI/R injury, which suggested that miR-432 augmented HIF-1α activation by increasing β-catenin expression. At the same time, we also found that the knockdown of β-catenin could not only significantly increase the rate of cardiomyocyte apoptosis (**Figure 4B**), the IA to AAR ratio (**Figure 4C**), and serum creatine phosphokinase (**Figure 4D**) induced by MI/R injury, but could also significantly reverse the decrease in the rate of cardiomyocyte apoptosis, the IA to AAR ratio, and serum creatine phosphokinase induced by miR-432 overexpression in the heart of mice at 24 hours after MI/R injury.

We aimed to determine whether HIF-1α was the executor of the protective effect exerted by miR-432 against MI/R injury. We regulated miR-432 expression in the heart tissues by transfecting them with miR-432 mixed with the transfection reagent, and regulated HIF-1α expression by transfecting the tissues with a siRNA specific to β-catenin (siHIF-1α) and negative control siRNA (sictrl), which was used as the negative controls. The results of the western blotting analysis indicated that siHIF-1α could significantly decrease the expression of HIF-1α in the heart tissues of mice at 24 hours after MI/R injury with or without miR-432 overexpression (**Figure 4E**). For the apoptosis-related proteins, HIF-1α knockdown could not only significantly increase the ratio of Bax/Bcl2 protein expression and cleaved-caspase 3 protein expression, but could also significantly reverse the decrease in the ratio of Bax/Bcl2 protein expression and cleaved-caspase 3 protein expression induced by miR-432 overexpression at 24 hours after MI/R injury (**Figure 4E**). We also found that HIF-1α knockdown not only significantly increased the rate of cardiomyocyte apoptosis (**Figure 4F**), IA to AAR ratio (**Figure 4G**), and serum creatine phosphokinase (**Figure 4H**) induced by MI/R injury, but also significantly reversed the decrease in the rate of cardiomyocyte apoptosis, the IA to AAR ratio, and serum creatine phosphokinase induced by miR-432 overexpression in the heart tissues of the mice at 24 hours after MI/R injury.

miR-432 acts on anti-oxidative stress after MI/R injury

In this study, we also investigated the effect of miR-432 expression on MI/R-induced oxidative stress. At 24 hours after MI/R injury, we harvested the hearts of the mice and detected levels of oxidative stress biomarkers. We found that MI/R injury significantly decreased the content of superoxide dismutase (SOD) (**Figure 5A**) and glutathione peroxidase (GSH-PX) (**Figure 5B**), but significantly increased the content of malondialdehyde (MDA) (**Figure 5C**) and $gp91^{phox}$ protein expression (**Figure 5D**). Then, we evaluated the effect of miR-432 on MI/R-induced oxidative stress by regulating miR-432

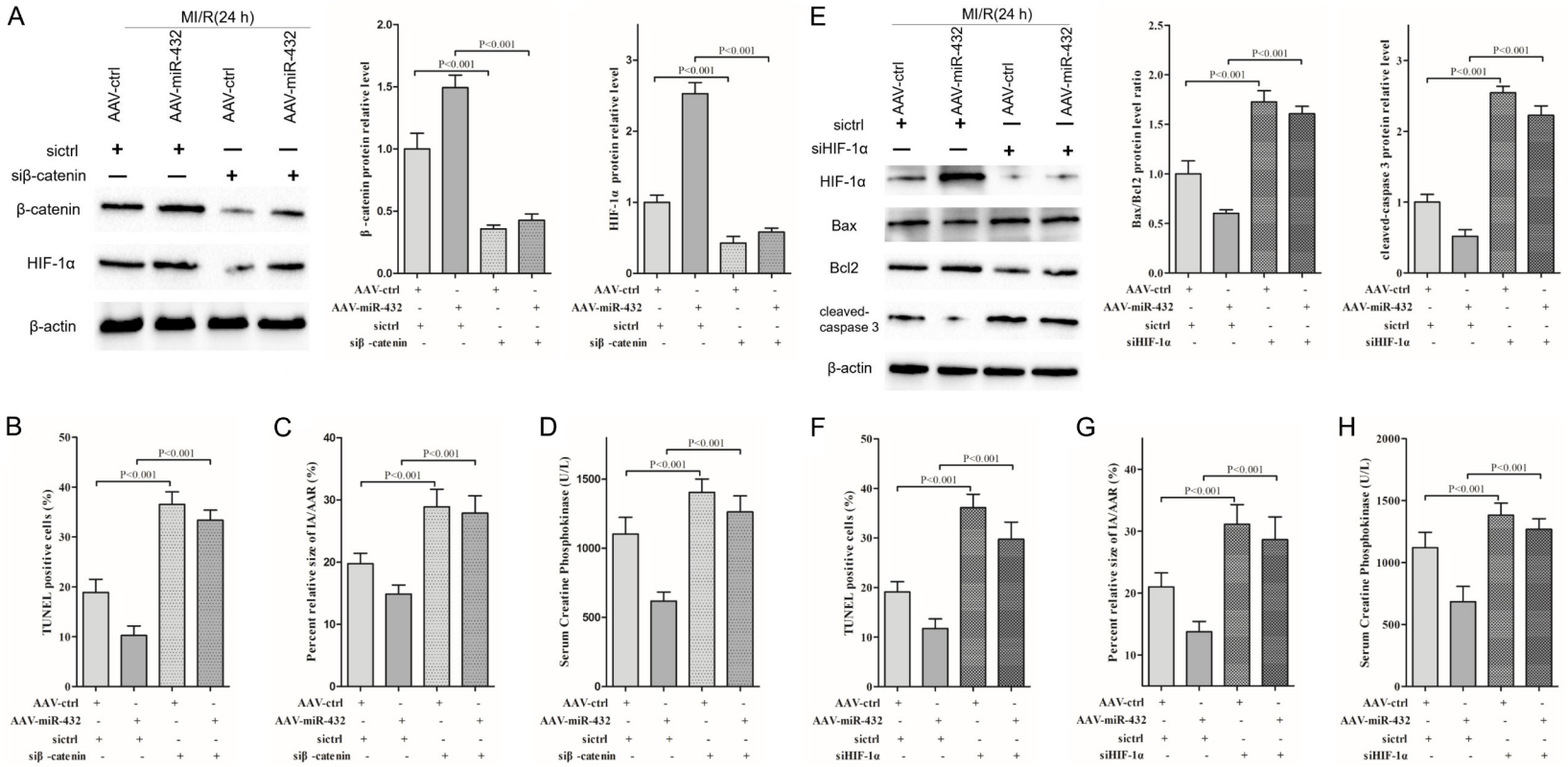

Figure 4. miR-432 decreases MI/R-induced apoptosis through β-catenin/HIF-1α pathway. (A-D) Mice were intra-myocardially transfected with miR-432 mixed transfection reagent or absence of transfection of control siRNA (siCtrl) or β-catenin siRNA (siβ-catenin) 48 hours prior to MI/R surgery. At 24 hours of MI/R injury, we euthanized mice to analysis β-catenin and HIF-1α protein expression (A), cardiomyocyte apoptosis (B), percent of infarct area (IA) to AAR (C) and the levels of serum of creating phosphokinase (D). (E-H) Mice were intra-myocardially transfected miR-432 mixed transfection reagent or absence of transfection of control siRNA (siCtrl) or HIF-1α siRNA (siHIF-1α) 48 hours prior to MI/R surgery. At 24 hours of MI/R injury, we euthanized mice to analysis the ratio Bax to Bcl2 and cleaved-caspase 3 protein expression (E), cardiomyocyte apoptosis (F), percent of infarct area (IA) to AAR (G) and the levels of serum of creating phosphokinase (H). 8 mice each group, *P* value was calculated by student's t test.

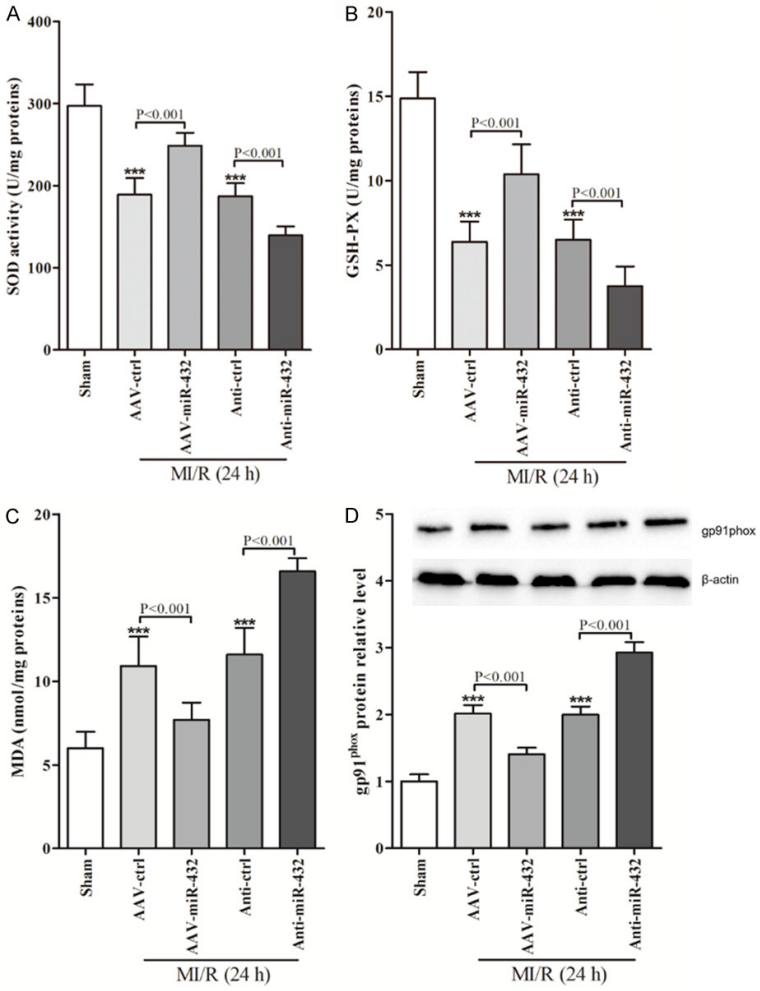

Figure 5. miR-432 decreases oxidative stress after MI/R injury. (A-D) miR-432 mixed transfection reagent was injected into the myocardium of mice to regulate miR-432 expression 48 hours prior to MI/R injury. 24 hours of MI/R injury, we harvested the mice heart to determine the levels of SOD (A), GSP-PX (B) and MDA (C) using spectrophotometer method, and to analyze gp-91^{phox} protein expression using western blot (D). 8 mice each group, *P* value was calculated by post-hoc comparisons, ***P<0.001 vs Sham group. SOD = Superoxide dismutase, GSH-PX = Glutathione peroxidase, MDA = Malondialdehyde.

expression in the heart of the mice, and found that miR-432 overexpression could significantly reverse the decrease in SOD and GSH-PX levels induced by MI/R injury. On the contrary, miR-432 knockdown could significantly decrease SOD and GSH-PX levels induced by MI/R injury. At the same time, miR-432 overexpression could significantly reverse the increase in MDA levels and gp91^{phox} protein expression induced by MI/R injury, while miR-432 knockdown could significantly increase already elevated MDA levels and gp91^{phox} protein expression induced by MI/R injury.

miR-432 exerts a protective effect against MI/R-induced oxidative stress by increasing the NRF2 content

The KEAP1-NRF2 axis, PI3K/Akt, eNOS, and mitochondrial mediated oxidative stress have been widely associated with MI/R injury [29, 30]. A previous study found that miR-432 directly targeted KEAP1 to induce NRF2 stabilization [24], and there is a binding site of miR-432 at position 1895-1901 of KEAP1 (**Figure 6A**). To investigate whether miR-432 could target KEAP1 in the cardiomyocytes, we first transfected the luciferase reporter containing WT or MUT KEAP1 sequences into H9c2 cells, and then regulated miR-432 expression to compare the activity of the luciferase reporter in H9c2 cells. The results showed that miR-432 overexpression could significantly decrease luciferase activity in the H9c2 cells transfected with the luciferase reporter containing WT-KEAP1 sequences, while miR-432 deletion could significantly reverse this effect (**Figure 6A**). At the same time, miR-432 overexpression significantly decreased the protein level of KEAP1, while miR-432 deletion significantly increase in the H9c2 cells (**Figure 6B**). Therefore, these results suggest that miR-432 acts as a target to inhibit KEAP1 protein expression in cardiomyocytes *in vitro*.

ROS change the conformation of KEAP1 by modifying its cysteine residues. Therefore, the decoupling of NRF2 from KEAP1 can be used to activate NRF2 mediated anti-oxidative stress. Therefore, in theory, miR-432 can play a role in anti-oxidative stress after MI/R injury by increasing the NRF2 content by targeting Keap1 expression. To test this hypothesis, we determined KEAP1 and NRF2 protein expression lev-

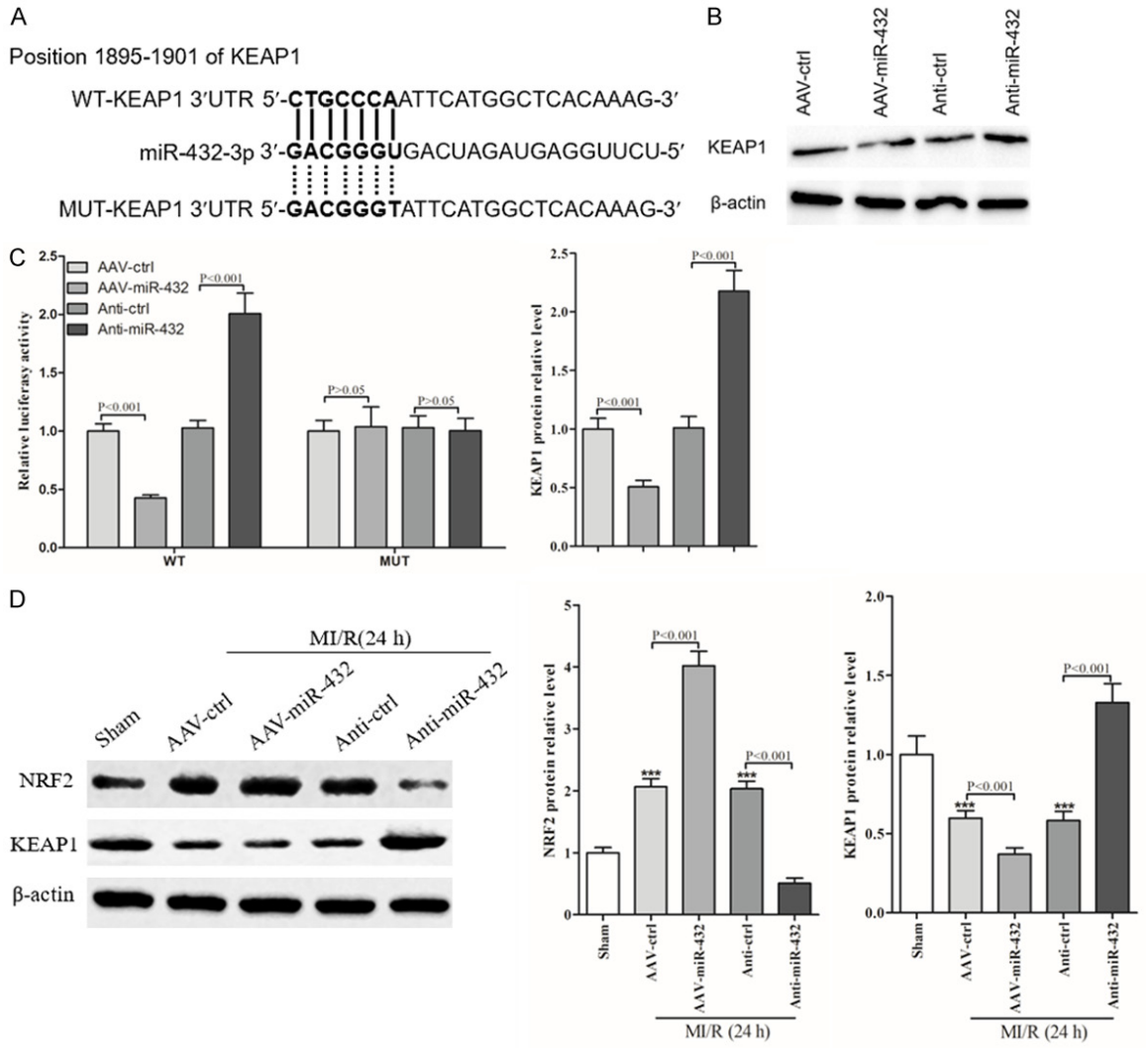

Figure 6. miR-432 augments NRF2 activation by targeting KEAP1. (A) Luciferase reporter containing WT or MUT KEAP1 sequences were transfected into H9c2 cells prior to transfect with miR-432 mixed transfection reagent, and harvested the activity of luciferase. 3 independent repetitions, *P* value was calculated by student's t test. (B) Western blot analysis of KEAP1 protein expression at 24 hours after transfecting with miR-432 mixed transfection reagent in H9c2 cells. 3 independent repetitions, *P* value was calculated by student's t test. (C, D) miR-432 mixed transfection reagent was injected into the myocardium of mice to regulate miR-432 expression 48 hours prior to MI/R injury. 24 hours of MI/R injury, we harvested the mice heart and used western blot method (C) to analyze the expression of NRF2 and KEAP1 protein (D). 8 mice each group, *P* value was calculated by post-hoc comparisons, ***P<0.001 vs Sham group.

els, and found that miR-432 overexpression could significantly decrease KEAP1 protein expression and increase NRF2 protein expression (**Figure 6C** and **6D**), while miR-432 deletion could significantly increase KEAP1 protein expression and decrease the NRF2 protein level at 24 hours after MI/R injury. Furthermore, we also found that NRF2 deletion could significantly reverse the decrease in gp91^{phox} protein expression (**Figure 7A** and **7B**) and MDA levels (**Figure 7C**) induced by miR-432 overexpression in the heart tissues of the mice at 24 hours after MI/R injury. NRF2 deletion significantly reversed the increase in SOD (**Figure 7D**) and GSH-PX levels (**Figure 7E**) in the heart tissues of the mice at 24 hours after MI/R injury. Importantly, NRF2 deletion could also significantly reverse the decrease in the IA to AAR ratio (**Figure 7F**) and serum creatine phosphokinase level (**Figure 7G**) induced by miR-432 overexpression in the heart tissues of the mice at 24 hours after MI/R injury.

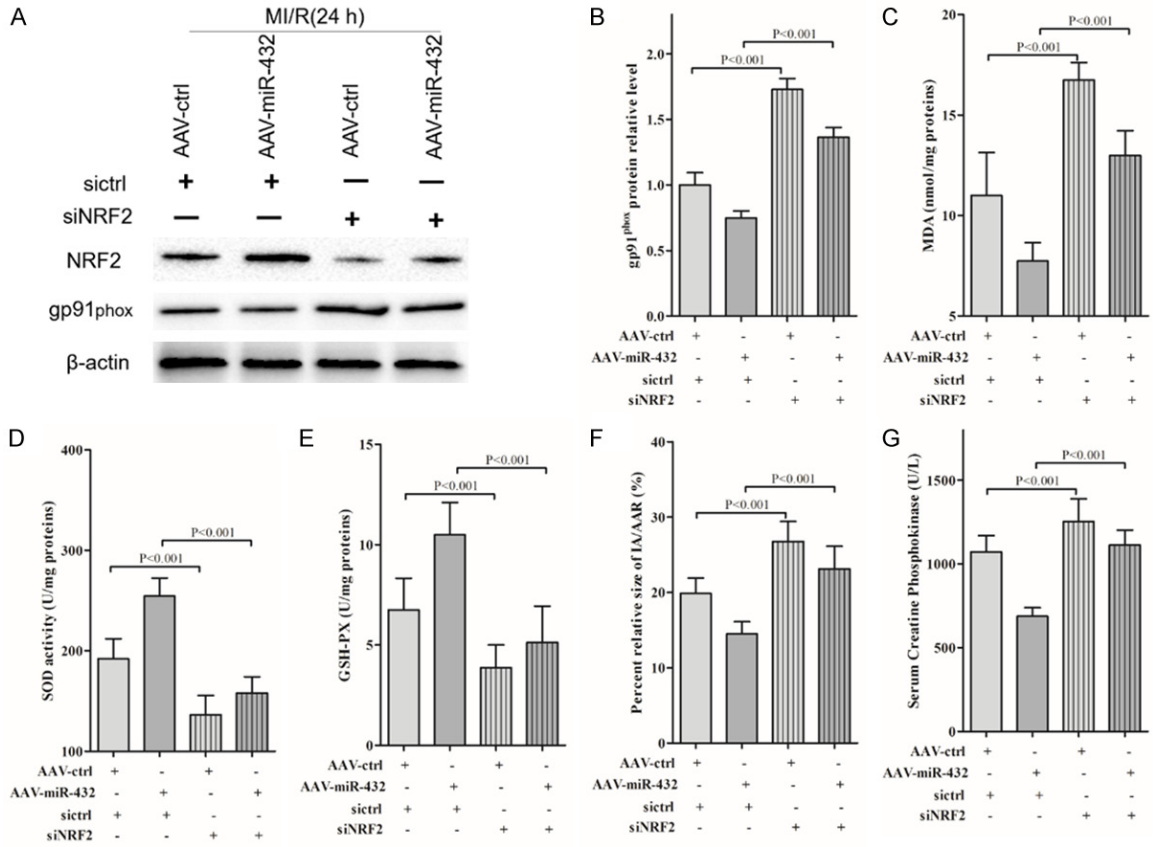

Figure 7. NRF2 deletion weakens the anti-oxidative stress effect of miR-432 after MI/R injury. (A-G) Mice were intra-myocardially transfected with miR-432 mixed transfection reagent or absence of transfection of control siRNA (siCtrl) or NRF2 siRNA (siNRF2) 48 hours prior to MI/R surgery. At 24 hours of MI/R injury, we euthanized mice to analysis NRF2 and gp91^{phox} protein expression (A, B), the levels of SOD (C), GSP-PX (D) and MDA (E), the percent of infarct area (IA) to AAR (F) and the levels of serum of creating phosphokinase (G). 8 mice each group, *P* value was calculated by student's t test. SOD = Superoxide Dismutase, GSH-PX = Glutathione Peroxidase, MDA = Malondialdehyde.

Discussion

In this study we found that miR-432 plays an important role in MI/R injury in mice. I/R injury increased miR-432 expression in the heart tissues during the first 24 hours but down-regulated from 24-96 hours after MI/R injury. Functionally, miR-432 overexpression significantly decreased the IA to AAR ratio and serum creatine phosphokinase level, while miR-432 knockdown significantly increased the IA to AAR ratio and serum creating phosphokinase level in the heart tissues at 24 hours after MI/R injury.

The Wnt signaling pathway is a relatively conservative signaling pathway that exerts important effects on ontogeny, cell apoptosis, and necrosis. The Wnt signaling pathway can be classified into two types based on whether the activation of the pathway involves β-Catenin, namedly, the classical/canonical pathway (Wnt/β-catenin signaling pathway) [31] or the non-canonical pathway (Wnt-Ca^{2+} signaling pathway and planar cell polarity signaling pathway) [32]. Wnt/β-catenin signaling has been demonstrated to be very important for various diseases, including in ischemia/reperfusion injury, possibly via the regulatory effect on apoptosis and necrosis [19, 33-35]. Chen et al. found that β-catenin activation decreased MI/R-induced apoptosis in mice hearts [19]. Other researchers have found that miR-432 suppresses bladder cancer cell apoptosis by targeting RBM5 through the β-catenin pathway [21] and also directly regulates Wnt/β-catenin in hepatocellular carcinoma (20). This study showed that miR-432 overexpression signifi-

cantly increased β-catenin expression and the p-GSK-3β/GSK-3β ratio, while miR-432 knockdown significantly decreased β-catenin protein expression and the p-GSK-3β/GSK-3β protein expression ratio at 24 hours after MI/R injury. When not activated, the coaxial protein of β-Catenin, adenomatous polyposis coli, GSK3, and casein kinase 1 form a multi protein complex that can degrade β-catenin to maintain a low level of β-catenin in the cells. When cells receive an exogenous stimulation signal, β-catenin is dissociated from the degradation protein complex, which leads to an increased β-catenin expression. Therefore, our results demonstrate that miR-432 activates Wnt/β-catenin signaling after MI/R injury.

It is well known that miR-432, a non-coding RNA, is not directly involved in the regulation of cell activities and must proceed through the regulation of target genes. However, there is no binding site for miR-432 in the β-catenin sequence. Based on previous study, we found that miR-432 is a target for inhibiting RBM5 expression *in vitro* and *in vivo*. RBM5 overexpression could not only significantly reverse the increase in β-catenin protein expression, but also significantly reverse the decrease in the rate of apoptosis in the mice heart induced by miR-432 overexpression at 24 hours after MI/R injury. Hypoxia-inducible factor 1α (HIF-1α), which mediates the adaptive response in the heart of MI/R injury has emerged as a key cardio-protective factor that can mediate the survival of cardiomyocytes [36, 37], while β-catenin has been found to decrease MI/R-induced cardiomyocyte apoptosis by activating HIF-1α after MI/R injury [19]. Therefore, to further investigate the molecular mechanism by which miR-432 mediates the survival of cardiomyocytes, we investigated the relationship between miR-432 and HIF-1α following MI/R injury. The results showed that miR-432 expression decreased the rate of MI/R-induced apoptosis through the β-catenin/HIF-1α pathway. It should be pointed out that inhibition of β-catenin/HIF-1α and NRF2 pathways should be simultaneously tested to see whether the cardioprotective effects of miR-432 can be fully reversed. However, due to limited experimental techniques, we were unsuccessful to inhibit β-catenin/HIF-1α and NRF2 pathways, and it does not seem to be a superposition of single pathway inhibition.

Conclusion

miR-432 is upregulated in heart tissues after MI/R injury and exerts a protective effect against MI/R injury. The specific mechanism by which the upregulation of miR-432 exerts a protective effect against MI/R injury proceeds through the activation of the β-catenin/HIF-1α pathway and augmentation of NRF2-mediated anti-oxidative stress.

Disclosure of conflict of interest

None.

Address correspondence to: Rui-Ying Su, Department of Cardiac Function Inspection, The Third Hospital of Hebei Medical University, No. 139, Ziqiang Road, Shijiazhuang 050051, Hebei, China. Tel: +86-311-88603500; Fax: +86-311-88603500; E-mail: surythirdhmu@163.com

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