

Research Article

The protective role of MiR-206 in regulating cardiomyocytes apoptosis induced by ischemic injury by targeting PTP1B

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MicroRNAs play essential roles in the regulation and pathophysiology of acute myocardial infarction (AMI). The purpose of the present study was to assess the expression signature of miR-206 in rat heart with AMI and the corresponding molecular mechanism. The expression of miR-206 significantly decreased in the infarcted myocardial areas and in hypoxia-induced cardiomyocytes, compared with that in the noninfarcted areas. Overexpression of miR-206 decreased cardiomyocytes apoptosis and the down-regulation of miR-206 increased cardiomyocytes apoptosis *in vitro*. In addition, overexpression of miR-206 in rat heart *in vivo* remarkably reduced myocardial infarct size and cardiomyocytes apoptosis. We identified that miR-206 had a protective effect on cardiomyocytes apoptosis with the association of its target protein tyrosine phosphatase 1B (PTP1B). Gain-of-function of miR-206 inhibited PTP1B expression and loss-of-function of miR-206 up-regulated PTP1B expression. Furthermore, overexpression of PTP1B significantly increased cardiomyocytes apoptosis. These results together suggest the protective effect of miR-206 against cardiomyocytes apoptosis induced by AMI by targeting PTP1B.

Introduction

Acute myocardial infarction (AMI) is one of the common but life-threatening cardiovascular conditions that the blood flow is abruptly blocked in the coronary arteries causing myocardial tissue damage [1]. There is an estimated 3–4 million people in the world affected by AMI and the case fatality is extremely high. The comprehensive understanding and elucidation of the molecular mechanisms underlying the complex process of AMI is of great significance for seeking novel therapeutic approaches for AMI.

MicroRNAs (miRNAs) are endogenous small non-coding RNA sequences that can bind to a complementary mRNA in the 3'UTR region and functions post-transcriptionally to negatively regulate target gene expression by degrading the targeted RNAs or inhibiting their translation [2]. So far there is an estimated 1000 miRNA genes in the human genome and they might regulate ~60% of protein-coding genes [3–5], which suggest the fundamental roles of miRNAs in major cellular functions in humans. Dysregulation of miRNAs has been identified to be involved in the pathogenesis of many different diseases such as tumorigenesis, neurological and myocardial diseases [6–8].

Accumulating evidence in recent studies has demonstrated the critical roles that miRNAs play in AMI and its related symptoms. For instance, an endothelial cell-enriched miR-126 has essential roles in protecting vascular integrity and angiogenesis [9,10]. And the expression levels of miR-126 are positively associated with incident myocardial infarction (MI) [11]. Dong et al. [12] revealed that miR-21 functions protectively in the early phase of AMI by targeting PDCD4 and reducing apoptosis of cardiac myocytes.

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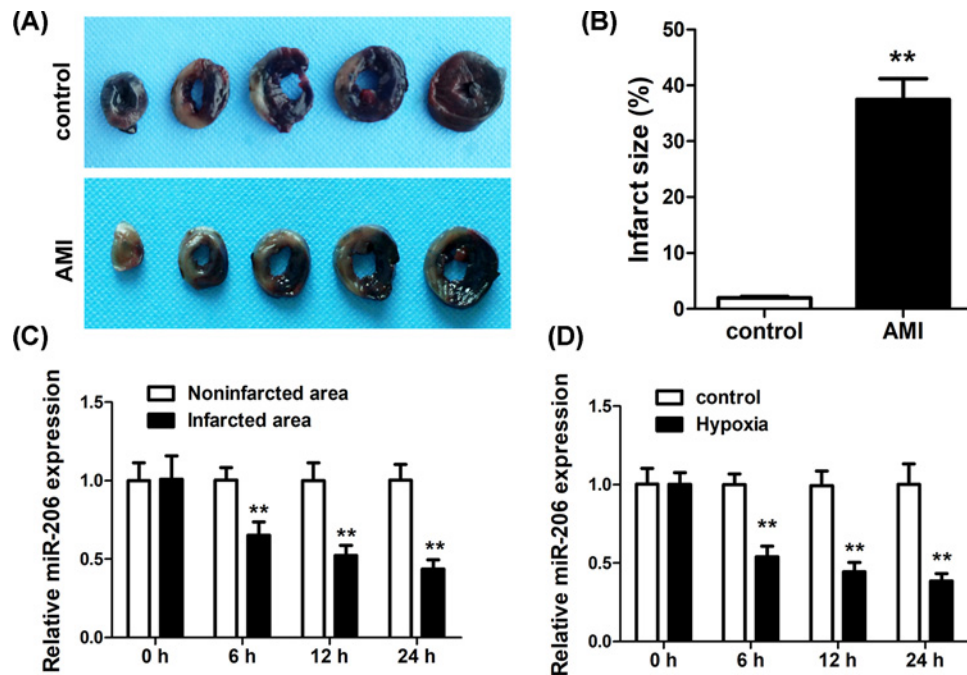


Figure 1. Expression levels of miR-206 in infarcted tissues and neonatal rat cardiomyocytes exposed to hypoxia

(A) Photographs showing representative slices for infarct myocardial tissues in the control and AMI rats. White areas represent the infarcted areas that were stained by both Evans blue and TTC. (B) Quantification data of the infarct area, which were calculated as the percentage of infarct size to the total area size in the control and AMI tissues. (C) qRT-PCR detection of the expression levels of miR-206 in the uninfarcted and infarcted tissues at indicated time points (0, 6, 12 and 24 h) after AMI. (D) qRT-PCR detection of the expression levels of miR-206 in neonatal rat cultured cardiomyocytes induced by hypoxia at indicated time points (0, 6, 12 and 24 h). U6 was used as an endogenous control. Data were presented as the mean \pm SD. **, statistically significant as $P < 0.01$.

MiR-499-5p has been reported to regulate the cell proliferation, differentiation and H_2O_2 -induced apoptosis of cardiomyocytes [13–15]. MiR-145 was previously reported to inhibit MI-induced apoptosis by regulating autophagy [16]. A recent study reported that the expression of miR-214 was highly up-regulated in elderly patients with AMI. It has inhibitory effect on apoptosis of cardiomyocytes by regulating its target genes [17]. The roles of miR-206 have been investigated in various diseases and it was identified in the regulation of cell proliferation and apoptosis, which suggest the potential roles of miR-206 in the modulation of pathophysiology of human diseases including AMI.

Protein tyrosine phosphatase 1B (PTP1B) is a ubiquitously expressed phosphatase and it has been identified as a pivotal modulator of insulin signaling pathways as it can dephosphorylate the insulin receptor and inactivate insulin signaling [18]. Recent studies also illustrated that increased activity of PTP1B was associated with the regulation of insulin sensitivity and contractile function, the pathophysiology of endothelial dysfunction, and cardiac dysfunction in heart failure [19–23]. The regulation of PTP1B by miR-210 was investigated in MI in mice and it showed that miR-210 directly targets PTP1B and the repression of PTP1B lead to improved angiogenesis and reduced apoptosis, which in turn improves the cardiac functions [24].

The aim of the present study was to investigate the impact of miR-206 on the regulation of cardiomyocytes apoptosis induced by AMI and the underlying mechanisms. We found that miR-206 has protective effects against AMI-induced apoptosis of cardiomyocytes by directly targeting PTP1B, which would be beneficial in elucidating the mechanisms underlying AMI.

Materials and methods

Experimental animals and generation of rat AMI models

Rat AMI model was generated with male Sprague–Dawley rats weighing 200–250 g (provided by Shanghai Research Center for Model Organisms, Shanghai, China) using left coronary artery (LCA) ligation procedure [25]. The rat model of AMI ($n=6$) was treated with an occlusion of the left anterior descending coronary artery and sealing by silk suture. And rats that received sham operation were used as controls ($n=6$). Animals were killed 2 weeks after the MI

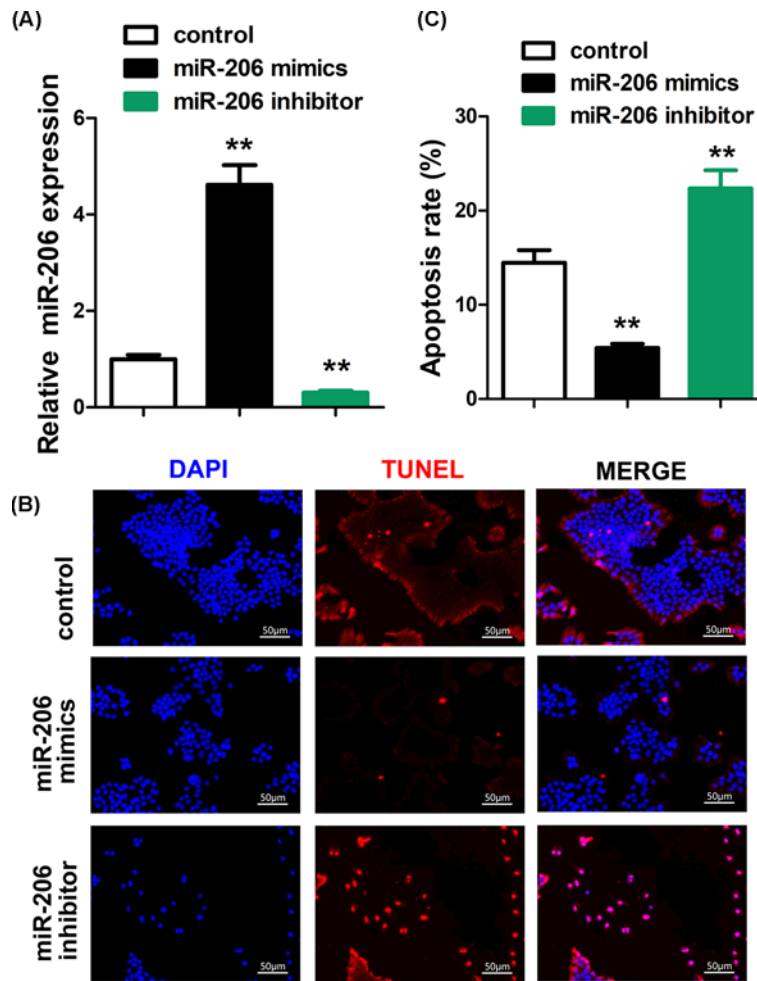


Figure 2. The effect of miR-206 on neonatal rat cardiomyocytes apoptosis induced by hypoxia

(A) qRT-PCR detection of the expression levels of miR-206 in transfected cardiomyocytes with miR-206 mimics, miR-206 inhibitor and the control, which were then induced by hypoxia. (B) Detection of cardiomyocyte apoptosis by TUNEL assay. (C) The number and rate of cardiomyocyte apoptosis were counted and calculated. Data were presented as the mean \pm SD. **, statistically significant as $P < 0.01$.

operation. All animal procedures were approved by the Animal Care and Use Ethic Committee at Central Hospital of Baoji City.

Measurement of infarcted, noninfarcted and border areas

To identify the infarcted and noninfarcted areas, the vena cava of the rats were injected with 3 ml of 1% Evans Blue dye. The ventricles of the heart were transversely cut into 2-mm slices and then immersed in 1% of 2,3,5-triphenyltetrazolium chloride (TTC) solution and incubated at 37°C for 30 min. Areas stained by Evans Blue in the myocardial slices was defined as the noninfarcted area. The area that was unstained was defined as the infarcted area. The area that was stained by TTC but unstained by Evans Blue was defined as the border area. Slices were photographed and the measurement of infarcted sizes was performed with the Osiris imaging software.

Culture of cardiomyocytes and induction of cell ischemia injury

The hearts of neonatal Sprague–Dawley rats (1–2 day old) were obtained and the ventricles were cut off and minced finely with micro-dissecting scissors. Ventricular cardiomyocytes were isolated and cultured in culture medium. Cardiomyocytes was then exposed to hypoxia in a chamber that was filled with 5% CO₂ and 95% N₂ to induce cell ischemia injury.

Plasmids transfections in cultured cardiomyocytes and overexpression of miR-206 *in vivo*

For the miR-206 knockdown, miR-206 overexpression, and PTP1B up-regulation, 100 nM of miR-206 inhibitor (purchased from GenePharma), 50 nM of miR-206 mimics (purchased from GenePharma), and 2 μ g of p-EZ-M02-PTP1B plasmid (purchased from FugenGen), respectively, was added to the culture medium for transfection using Lipofectamine™ 2000 (Invitrogen) following the manufacturer's protocol.

For the overexpression of miR-206 *in vivo*, miR-206 agomir (RiboBio) was introduced into rat hearts using the procedure described by Hajjar et al. [26]. In brief, rats were anesthetized and the pericardium was opened. Aorta and pulmonary arteries were identified and followed with aortic clamping. A catheter was advanced to the aortic root through the apex of the left ventricle (LV). And a 200- μ l solution that contains 10 nM control or miR-206 agomir (RiboBio) suspension in sterile PBS was injected through the catheter. Aortic clamping remained so the solution was circulated and the heart was perfused. Clamps were released after 10 s and the chest was closed.

qRT-PCR

Total RNA was extracted from tissues using TRIzol (Invitrogen) following the manufacturer's protocol. Total RNA was then reversely transcribed into cDNA using the M-MLV reverse transcriptase (Promega). The Bulge-Loop™ specific RT-Primers (RiboBio) and random primers (Promega) were used for miR-206 and PTP1B, respectively. qRT-PCR was performed using the SYBR Green reagents (Invitrogen) on the Applied Biosystems Prism® 7900HT Sequence Detection System. Either GAPDH or U6 was used as internal control. Relative expression level was calculated via the $2^{-\Delta\Delta C_t}$ method.

Detection of apoptosis by TUNEL assay

Cardiomyocytes that were on coverslips placed into 24-well plate and 8- μ m-thick sections of rat frozen heart were fixed in 4% paraformaldehyde solution. Apoptosis occurs in cultured cardiomyocytes and in heart sections were measured by the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining method [27] using the *In Situ* Cell Death Detection Kit (Roche) following the manufacturer's protocol. Samples were visualized under a fluorescence microscope and the numbers of total cells and TUNEL-positive cells were counted.

Western blot

Total protein was isolated from cultured cardiomyocytes cells with radioimmunoprecipitation assay (RIPA) buffer containing the protease inhibitor cocktail (Pierce). Protein concentration was measured by BCA Protein Assay Kit (Pierce). Equal amount of each protein sample was subjected to 8% SDS/PAGE and then transferred to polyvinylidene fluoride (PVDF) membrane (Millipore) for 30 min. Western blot analysis was conducted using anti-PTP1B antibody (1:1000, Abcam). An anti-GAPDH antibody (1:1000, Abcam) was used as a loading control. Protein signals were detected by enhanced chemiluminescence (ELC).

Luciferase assay

A fragment of the 3' UTR of PTP1B that contains the predicted binding site for miR-206 was amplified and cloned into a psiCHECK™ luciferase reporter vector (Promega). PTP1B 3' UTR mutant luciferase reporter constructs were subsequently constructed using Site-Directed Mutagenesis Kit (SBS Genetech). The vectors were named as PTP1B 3' UTR-wt (the wild-type) and PTP1B 3' UTR-mut (the mutant). The miR-206 mutant construct was constructed by introducing mutations into the miR-206 binding site with the Site-Directed Mutagenesis Kit (SBS Genetech). The construct was named as miR-206 mut. When the cell density reached ~50%, the two vectors were co-transfected with miR-206 mimics, inhibitor, mutant or control (RiboBio) into the cardiomyocytes using Lipofectamine™ 2000 (Invitrogen). Fresh medium was changed 6 h after the transfection and cells were cultured for 48 h. A Dual Glo™ Luciferase Assay System (Promega) was used to measure the luciferase activity, which was normalized to the activity of the *Renilla* luciferase expressing vector pRL-TK (Promega) that was used as control.

Statistical analysis

All statistical data analyses were performed using SPSS16.0 software (SPSS Inc.). Data values were presented as mean \pm standard deviation (SD). The differences between groups were determined using two-tail unpaired Student's *t* test. Differences with $P < 0.01$ were considered as statistical significance.

Results

Expression of miR-206 decreases in both infarcted myocardial tissues of AMI rat and neonatal rat cardiomyocytes exposed to hypoxia

To investigate the role of miR-206 in myocardial injury in rat, we first compared the AMI induced myocardial damage in the normal and AMI rat tissues. As shown in Figure 1A,B, the infarct area volume size was significantly increased in the AMI tissues ($P < 0.01$). The expression levels of miR-206 in uninjured and injured myocardial tissues were then quantified by qRT-PCR. As shown in Figure 1C, there was no significant difference between the uninjured and injured myocardial tissues in the Sham-operated control rats. While at 6, 12 and 24 h after AMI, the relative expression levels of miR-206 were significantly lower in the injured tissues than that in the uninjured tissues at ($P < 0.01$). The expression levels of miR-206 in cultured neonatal rat cardiomyocytes that were exposed to hypoxia were also assessed by qRT-PCR. As shown in Figure 1D, compared with the cardiomyocytes that were not induced by hypoxia, the relative expression level of miR-206 significantly decreased in cardiomyocytes at 6, 12 and 24 h after exposure to hypoxia. These results revealed that the expression of miR-206 decreases in both infarcted myocardial tissues of AMI rat and neonatal rat cardiomyocytes that were exposed to hypoxia.

MiR-206 inhibits hypoxia-induced apoptosis of cultured neonatal rat cardiomyocytes

The effect of miR-206 on neonatal rat cardiomyocyte apoptosis induced by hypoxia was detected by transfecting cultured cardiomyocytes with miR-206 mimics, miR-206 inhibitor and the negative control, followed with the induction of hypoxia. qRT-PCR results revealed that the miR-206 mimics remarkably increased ($P < 0.01$), while the miR-206 inhibitor remarkably decreased the expression of miR-206 in cardiomyocytes ($P < 0.01$) (Figure 2A). In addition, the effect of hypoxia-induced apoptosis on cardiomyocytes was demonstrated by TUNEL assay. As shown in Figure 2B,C, the number of TUNEL-positive cells in the miR-206 mimics cardiomyocytes was significantly lower ($P < 0.01$), while it was significantly higher in the miR-206 inhibitor cardiomyocytes ($P < 0.01$) compared with the control group. These results indicated that miR-206 could inhibit the hypoxia-induced apoptosis in cultured cardiomyocytes.

Overexpression of miR-206 protects heart against MI and cardiomyocytes apoptosis in *in vivo* rats

To assess the involvement of miR-206 in AMI, miR-206 agomir was delivered into rat hearts to overexpress miR-206 *in vivo*. As shown in Figure 3A, the expression of miR-206 was significantly up-regulated in rat hearts injected with miR-206 agomir ($P < 0.01$). And the size of myocardial infarct is remarkably reduced in rat hearts that were injected with miR-206 agomir compared with the control ($P < 0.01$) (Figure 3B,C). Cardiomyocytes apoptosis was also detected by TUNEL assay of infarcted heart sections (Figure 3D). As shown in Figure 3E, apoptosis rate of the miR-206-overexpression cardiomyocytes was significantly lower in the miR-206 agomir hearts compared with the control ($P < 0.01$). These results together demonstrated the protective effect of overexpressing miR-206 in rat heart by inhibiting both MI and cardiomyocytes apoptosis.

PTP1B is a direct target gene of miR-206

To further illustrate the molecular mechanisms underlying the protective role of miR-206 against MI and cardiomyocytes apoptosis, we performed bioinformatics analysis to find potential targets of miR-206 and found a putative binding site for miR-206 in the 3' UTR region of the *PTP1B* gene (Figure 4A). To verify this prediction, luciferase reporter assay was conducted by cloning a fragment of the wild-type or mutant 3' UTR of *PTP1B* in the predicted binding site into the luciferase gene vector, followed by co-transfection with miR-206 mimics, inhibitor, mutant, or the control. As shown in Figure 4B, overexpression of miR-206 significantly decreased the luciferase activities of the vector with the wild-type *PTP1B* 3' UTR ($P < 0.01$) but not in the mutant. And inhibition of miR-206 significantly increased the luciferase activities of the wild-type ($P < 0.01$) but not in the mutant. And the mutated form of miR-206 showed no difference in the luciferase activities between the wild-type *PTP1B* 3' UTR and the mutant (Figure 4B). Furthermore, the expression levels of *PTP1B* mRNA and protein were also assessed by qRT-PCR and Western blotting analysis, respectively. The results showed that overexpression of miR-206 significantly decreased the *PTP1B* mRNA levels (Figure 4C) and *PTP1B* protein expression (Figure 4D,E) in cardiomyocytes compared with that in the control ($P < 0.01$). In contrast, inhibition of miR-206 significantly increased *PTP1B* mRNA and protein expression ($P < 0.01$). These results together demonstrated that miR-206 directly targets and inhibits the expression of *PTP1B* in cardiomyocytes.

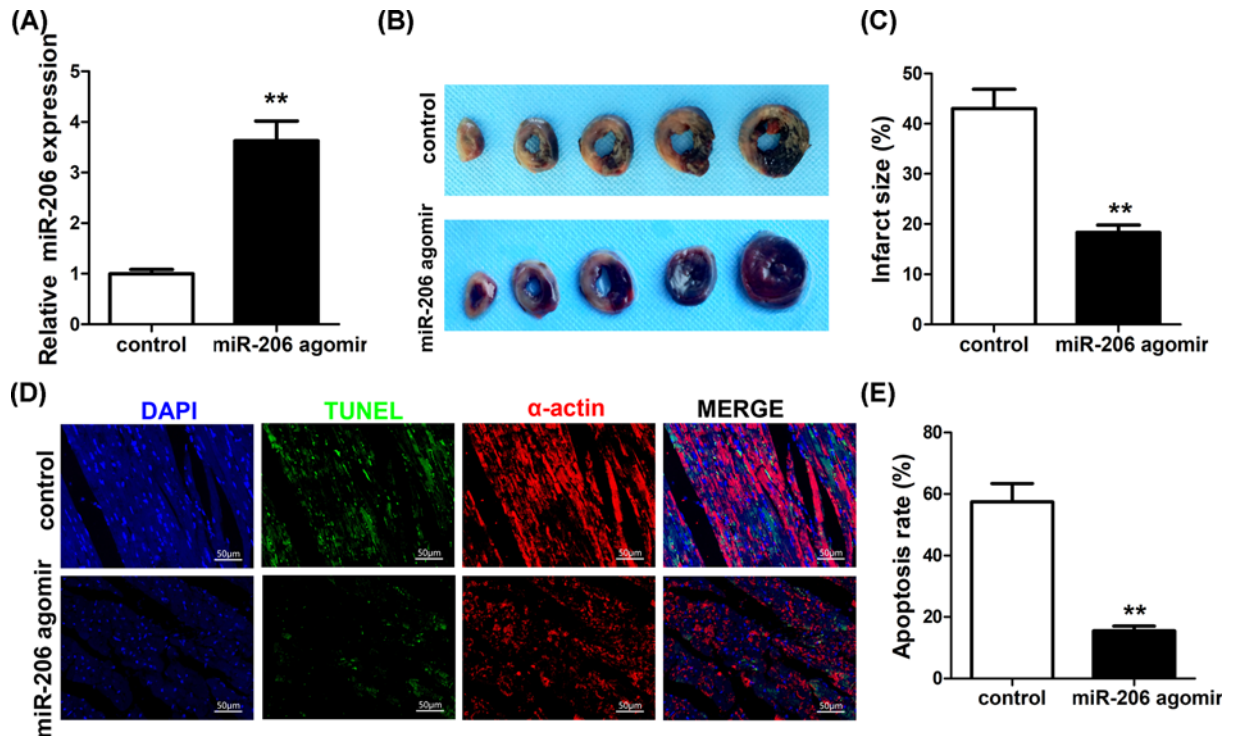


Figure 3. The role of miR-206 overexpression in protecting rat heart against MI and cardiomyocyte apoptosis

(A) qRT-PCR detection of the expression levels of miR-206 in rat hearts injected with miR-206 agomir or the control. (B) Photographs showing representative slices for rat hearts injected with miR-206 agomir or the control. White areas represent the infarcted areas that were stained by both Evans Blue and TTC. (C) Quantification data of the infarct area, which were calculated as the percentage of infarct size to the total area size in the control and miR-206 agomir hearts. (D) Detection of cardiomyocyte apoptosis of infarcted heart sections by TUNEL assay. (E) The rate of cardiomyocyte apoptosis was calculated in the control and miR-206 agomir hearts. Data were presented as the mean \pm SD. **, statistically significant as $P < 0.01$.

PTP1B is involved in miR-206-mediated apoptosis of cardiomyocytes

To further understand the mechanism of miR-206 in the apoptosis of cardiomyocytes, we investigated whether it mediated apoptosis by targeting PTP1B. The miR-206 mimics and PTP1B plasmid or PTP1B empty vector were co-transfected in the cultured cardiomyocytes. Overexpression of PTP1B significantly increased the protein expression level of PTP1B compared with the empty vector control as shown in Western blotting ($P < 0.01$) (Figure 5A,B). TUNEL assay of cultured cardiomyocytes also showed significantly increased cell apoptosis in cardiomyocytes transfected with PTP1B plasmid than that with the empty vector control (Figure 5C,D). These results suggest that PTP1B is involved in miR-206-mediated anti-apoptotic effect on cardiomyocytes.

Discussion

AMI is a complex process and involves dysregulations of a variety of genes [28]. And the essential roles that miRNAs are playing in the regulation and pathophysiology of AMI have been demonstrated in various studies. The current study demonstrated that miR-206 expression decreased in both infarcted myocardial tissues of AMI rat and neonatal rat cardiomyocytes exposed to hypoxia. MiR-206 protects cardiomyocytes against MI and apoptosis by targeting PTP1B. Progresses that have been made in studying the key regulators investigated in the present study will be discussed below.

MiRNAs modulate MI by regulating cell autophagy, necroptosis and apoptosis [29]. Increasing numbers of miRNAs have been identified as aberrantly expressed and are important regulators in MI. Therefore, future studies on miRNAs as novel therapeutic targets or biomarkers for AMI and other diseases would be challenging but also encouraging.

MiRNAs are not only functioning as key regulators in various signaling pathways involved in human disease development [30], but could also be serving as valuable diagnostic biomarkers of diseases. The critical roles that miRNAs play in the initiation and progression of cardiovascular disease have been well reviewed before [31,32]. Wronska et al.

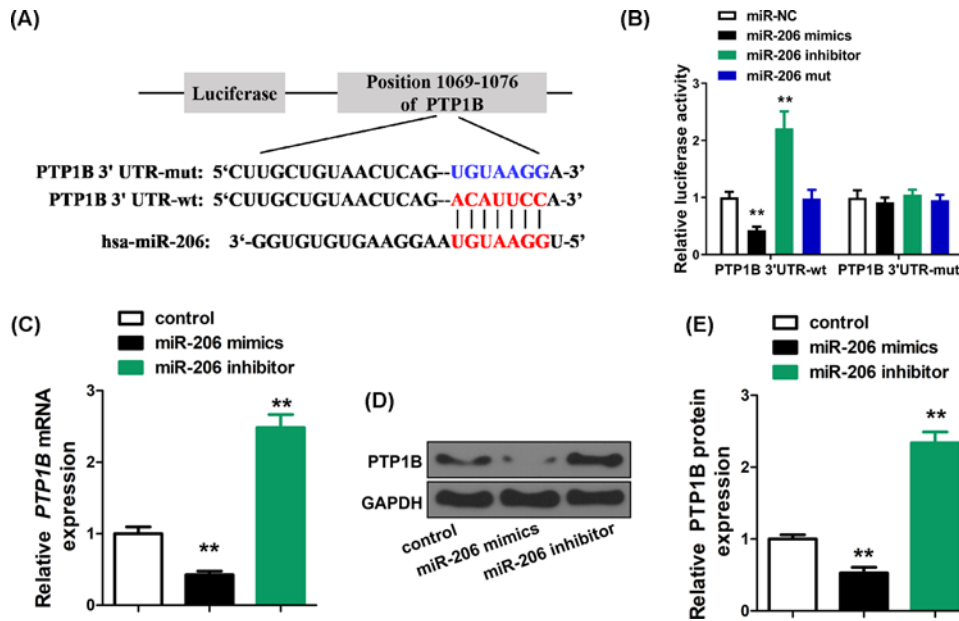


Figure 4. MiR-206 directly targets PTP1B

(A) Predicted binding site of miR-206 in the 3' UTR region of PTP1B. (B) The relative luciferase activity in cultured cardiomyocytes by cloning a fragment of the wild-type (PTP1B 3' UTR-wt) or mutant (PTP1B 3' UTR-mut) 3' UTR of PTP1B into the luciferase reporter vector and followed by co-transfection with miR-206 mimics, inhibitor or the control. (C) qRT-PCR detection of expression of PTP1B in cardiomyocytes. (D) Expression of PTP1B as determined by Western blot. GAPDH was used as an endogenous control. (E) Quantitative data of PTP1B protein expression. Data were presented as the mean \pm SD. **, statistically significant as $P < 0.01$. MiR-128-1 promoter mutated at putative p53 binding sites (Mut).

[33] recently conducted a systematic overview of the functions of miRNAs involved in the physiology, pathophysiology, diagnosis and treatment of cardiovascular diseases. The involvement of miRNAs in many of AMI studies was also reviewed recently [12]. The regulations and functions of miR-206 in skeletal muscle cell proliferation, differentiation, regeneration and the underlying molecular mechanisms have been well illustrated [34]. Pan et al. [35] showed that overexpression of miR-206 targets ANXA2 via repression of AKT signaling pathway and inhibits osteosarcoma cell proliferation, migration and invasion, and promotes apoptosis. A recent study revealed that the expression of serum miR-206 was decreased in hyperthyroidism patients and it is involved in the mediation of thyroid hormone regulation in human hepatoblastoma cells [36]. The protective effects of miR-206 on inhibition of cell proliferation and promotion of cell apoptosis have also been widely studied in various human diseases including knee articular osteoarthritis, prostate cancer, cervical cancer, triple negative breast cancer and etc. [37–40]. All these findings suggest the essential roles of miR-206 in the modulation of pathophysiology of human diseases including AMI. In our current study, we determined the protective effects of miR-206 on AMI by inducing ischemia injury in a cardiac cell model. We also applied gain-of-function and loss-of-function analyses to verify the role of miR-206 in regulating cardiomyocytes apoptosis. We confirmed that miR-206 has anti-apoptotic effect on cultured cardiomyocytes with hypoxia-induced injury *in vitro*. Besides, we also performed *in vivo* assay in rat AMI model to verify our findings of the anti-apoptotic effect of miR-206, in which overexpression of miR-206 reduced the myocardial size and cardiac cell apoptosis in rat hearts.

PTP1B as a phosphatase has been well characterized in modulating the insulin signaling pathway in various diseases. It was also identified as a key mediator for metabolism and oncogenesis [41]. A recent study pointed out that PTP1B could also be a potential target to modulate cardiac insulin sensitivity and contractile function in the failing heart [22]. It is not surprising that PTP1B and its protein substrate targets together regulate a complex signaling network. However, the upstream regulations that modulate PTP1B expression need to be further elucidated. Our present study validated that miR-206 directly targets the 3' UTR of PTP1B to regulate hypoxia-induced cardiomyocytes apoptosis. The present study extended our knowledge in illustrating the effects of miR-206 and PTP1B in the pathophysiology of AMI. However, there are still more functional data needed to fully understand the cellular

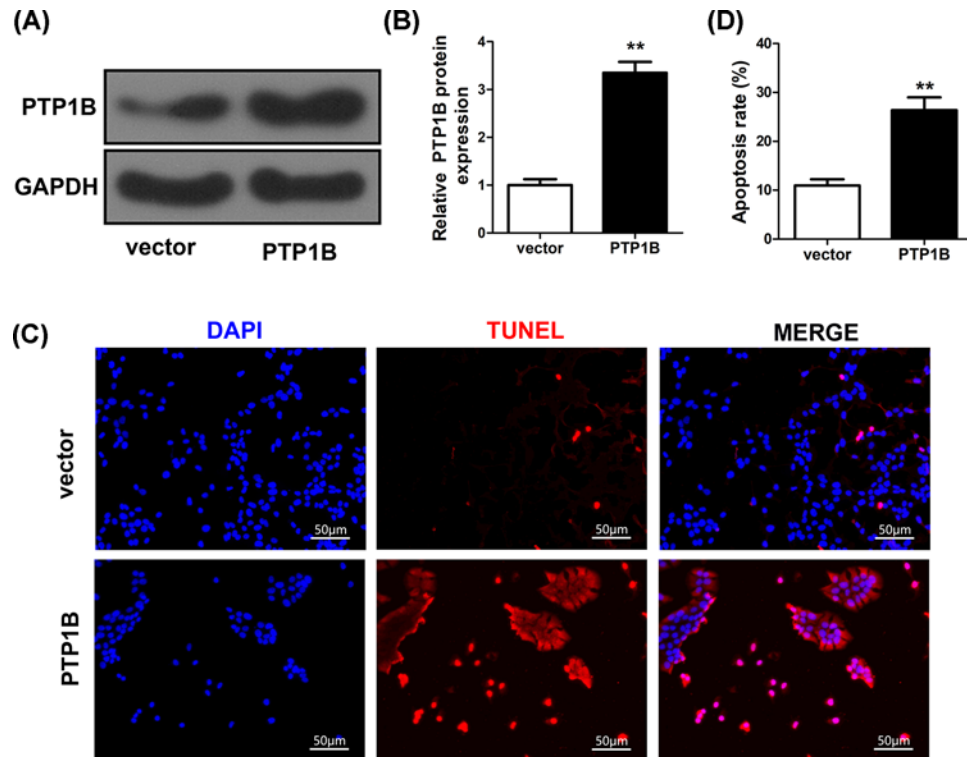


Figure 5. PTP1B is involved in the effect of miR-206-mediated cardiomyocytes apoptosis

(A,B) Relative protein expression levels of PTP1B in cardiomyocytes that were co-transfected with either PTP1B plasmid or empty vector control as determined by Western blot. GAPDH was used as an endogenous control. (C) Detection of apoptosis of cardiomyocyte transfected with either PTP1B plasmid or empty vector control by TUNEL assay. (D) The number and rate of cardiomyocyte apoptosis were counted and calculated. Data were presented as the mean \pm SD. **, statistically significant as $P < 0.01$.

mechanisms in miR-206-mediated effects on AMI, which could be our future research insight for seeking novel therapeutic targets for cardiovascular disease. Besides, fully profiling and understanding of the comprehensive signaling network of miRNAs regulating AMI are also needed in order to reduce any possible imprecision in predicting the disease-associated key regulators.

In summary, this work has important implications for uncovering the mechanisms under AMI and suggests the potential use of modulation of miR-206 and PTP1B-mediated signaling pathway as a molecular therapeutic strategy in AMI early treatment.

Data Availability Statement

The data that support the findings of the present study are available from the corresponding author upon reasonable request.

Ethics Approval

All animal procedures were approved by the Animal Care and Use Ethnic Committee at Central Hospital of Baoji City.

Author Contribution

Yejun Yan was responsible for experimental work, manuscript writing, literature search and data analysis. Xia Wang and Xiaodong Liu were responsible for data analysis and statistical analysis. Hongwei Dang and Xin Zhang were responsible for research design and manuscript editing. All authors read and approved the final manuscript.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

AMI, acute myocardial infarction; ANXA2, Annexin A2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MI, myocardial infarction; miRNA, microRNA; PDCD4, Recombinant Human Programmed Cell Death 4; PTP1B, protein tyrosine phosphatase 1B; qRT-PCR, Quantitative reverse transcriptase PCR; TTC, 2,3,5-triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotide transferase dUTP nick-end labeling.

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