MiR-134-5p Regulates Myocardial Apoptosis and Angiogenesis by Directly Targeting KDM2A After Myocardial Infarction

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Summary

MiR-134-5p was found to have potential diagnostic value for myocardial infarction (MI), but its biological role in MI has not been reported. In this study, MI mouse model was established. Quantitative real-time PCR (qRT-PCR) and western blot were used to measure the expression of miR-134-5p, lysine demethylase 2A (KDM2A), and vascular endothelial growth factor (VEGF). Dual-Luciferase reporter (DLR) assay was used to explore the relationship between miR-134-5p and KDM2A. The influence of miR-134-5p on cardiomyocytes apoptosis was detected using methyl thiazolyl tetrazolium (MTT) assay. The results revealed that miR-134-5p was highly expressed in infarction tissues of MI mice. Knockdown of miR-134-5p inhibited hypoxia/reoxygenation (H/R)-induced cardiomyocyte apoptosis. In addition, KDM2A was the target gene of miR-134-5p and negatively regulated by miR-134-5p. The promotion effect on the protein level of KDM2A and VEGF induced by miR-134-5p inhibitor can be reversed by shKDM2A in cardiomyocytes. Further, silencing of miR-134-5p promoted myocardial angiogenesis and inhibited myocardial apoptosis via upregulating KDM2A in MI mice. Taken together, our research revealed that knockdown of miR-134-5p increased KDM2A expression, thereby suppressing myocardial apoptosis and promoting myocardial angiogenesis.

Key words: Vascular endothelial growth factor (VEGF), Hypoxia/reoxygenation (H/R), Mice, Cardiomyocyte

Myocardial infarction (MI) is the main cause of high disability and mortality in patients with cardiovascular diseases worldwide. The mechanism of myocardial repair after MI is very complicated. At present, MI treatment can only relieve the symptoms but cannot solve the problem of myocardial cell loss from the root. As a result, the incidence and mortality of heart failure after MI continue to rise. Studies have confirmed that cardiomyocyte apoptosis is an important cause of ventricular remodeling, cardiac insufficiency, and arrhythmia after MI. Myocardial angiogenesis is a key process for ventricular remodeling after MI. Inhibition of cardiomyocyte apoptosis and promotion of angiogenesis can improve the prognosis of patients with MI. Therefore, effectively inhibiting cardiomyocyte apoptosis and promoting angiogenesis in post-MI are of great significance for alleviating ventricular pathological remodeling, improving cardiac function, and inhibiting the occurrence and development of heart failure.

As a kind of endogenous noncoding RNA with extensive regulatory functions, microRNAs (miRNAs) can regulate the expression of target genes by binding to the 3’-untranslated region (3’-UTR) of target genes and then participate in a series of life processes such as apoptosis, proliferation, and differentiation. Broad evidence has demonstrated that miRNAs widely exist in myocardial tissues and paly a regulatory role in the pathophysiological processes of cardiovascular diseases, including MI. Some miRNAs have been shown to promote or inhibit cardiomyocyte apoptosis through regulating the expressions of related target genes, thus, participating in the occurrence and development of MI. MiR-134-5p is a kind of miRNAs, which was indispensable for cell differentiation, proliferation, and migration. It was reported that plasma miR-134-5p level was significantly increased in early stage of AMI, which had potential diagnostic value for the early phase of AMI. However, the underlying mechanisms of miR-134-5p in MI have not been explored.

Lysine demethylase 2A (KDM2A) is a demethylase, which plays an important role in transcriptional cell proliferation, angiogenesis, tumorigenesis regulation, and artificial induced pluripotent stem cell technology. Bioinformatics software (TargetScan) predicts that KDM2A may be a target gene of miR-134-5p. Thus, we speculate that the role of miR-134-5p in MI may be related to KDM2A.

In this study, we investigated the expression and the underlying mechanisms of miR-134-5p and KDM2A in MI, aiming to find new targets for MI treatment.

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Methods

MI mouse model: We purchased 40 C57BL/6 mice (male, 6-8 weeks, 25-28 g) from Guangdong Medical Laboratory Animal Center. The Ethics Committee of Chongming Branch Xinhua Hospital affiliated to Medical College of Shanghai Jiaotong University approved all animal experimental procedures. MI model was established as described previously.21 Mice were anesthetized using 4%-5% chloral hydrate (300-450 mg/kg). The 6-0 Prolene suture was used to ligate the left anterior descending coronary artery. The model was considered successful when the left ventricle anterior wall turned white. All procedures except ligation were performed on mice in sham group. Two days before MI operation, mice in MI+miR-134-5p antagonir group and MI+NC (negative control) antagonir group were treated with miR-134-5p antagonir or NC antagonir by intramyocardial injection. Twenty-four hours after MI operation, serum samples of mice were collected for lactate dehydrogenase (LDH) release assay, which was performed using a multifunction detector (Thermo Fisher Scientific, Waltham, MA, USA). In addition, myocardial tissues including infarction area were collected for the following experiments.

Triphenyltetrazolium chloride (TTC) staining: The infarct size was detected by performing TTC staining assay. The left ventricle was collected and cut into 1-2 thick slices. Then, the slices were incubated in TTC solution (1%) for 15 minutes at 37°C. When the non-infarcted area turned was stained and the infarction area was red white, the slices were fixed in 10% paraformaldehyde for half an hour. Next, a microscope was used to observe the stained slices, and the images were captured by Image-Pro Plus 6.0 software.

Primary cultures of cardiomyocytes: The experiment was performed as previously described.22 The hearts of neonatal mice were collected and kept in cold DMEM (Thermo Fisher Scientific). The ventricles were cut into 4-6 uniform parts and digested with collagenase II. Then, the supernatant of the digested solution was collected and added to an EP tube containing 100% FBS. The above steps were repeated seven to eight times until the myocardial tissues were completely digested. The digest gaster was collected into an EP tube and spun at 1500 rpm for 5 minutes. The supernatant was removed, and DMEM containing 10% FBS was added to resuspend cells. One hour later, the unattached cardiomyocytes in suspension were collected and placed in a culture plate coated with fibronectin. Twenty-four hours later, cardiomyocyte cultures were used.

Cell treatment: MiR-134-5p inhibitor (inh) and its NC inh, as well as shKDM2A and its negative control (shNC), were procured from Shanghai GenePharma Co., Ltd. (China). Lipofectamine 2000 (Invitrogen, Carlsbad, USA) was used to perform cell transfections following the manufacturer’s protocol. The method of cells hypoxia/reoxygenation (H/R) treatment: cardiomyocytes were maintained in serum-free DMEM and cultured in anoxic tank. To create the hypoxia model, Genbox anaerobic gas bags were placed in the anoxic tank. After 10 hours, cardiomyocytes were collected and cultured in complete medium for 2 hours. Cells in control (Con) group were cultured under normal conditions. LDH level in the cell culture medium was detected using LDH Release Assay Kit (Beyotime, Shanghai, China) following the manufacturer’s instructions.

Methyl thiazolyl tetrazolium (MTT) assay: Cell viability was determined by MTT assay. Firstly, cells were seeded into 96-well plates (1 × 10^4 cells/mL) in each well and cultured for 3 days. Then, 5 mg/mL MTT (15 μL) was added into each well. Four hours later, the supernatants were removed carefully. To dissolve the resultant formazan crystals, DMSO (150 μL/per well) was added to each well. A spectrophotometer was used to measure the absorption at 490 nm.

Quantitative real-time PCR (qRT-PCR): Trizol reagent (Invitrogen) was used to extract total RNAs from cells or tissues following the manufacturer’s protocol. cDNA reverse using Transcripase Kit (Takara, Otsu, Japan) and SYBR Green PCR Master Mix (Takara, Otsu, Japan) with the ABI StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used for qRT-PCR. The 2^{ΔΔCt} method was used to calculate miR-134-5p expression. U6 was used for miRNA control. Each sample was performed three times. The sequence of primers for miR-134-5p and U6 was listed in the Supplemental Table.

Western blot: Total protein was extracted from cells or tissues using RIPA lysis buffer (Beyotime, Beijing, China). A BCA protein assay kit (Beyotime) was used to measure the protein concentrations following the manufacturer’s protocols. SDS-PAGE (12%) and PVDF membranes were used for separating and transferring the protein samples. Skim milk (5%) was used to block the membranes for 2 hours at room temperature. After that, the membranes were incubated with primary antibodies at 4°C overnight, which include anti-Bcl2 antibody (1:1000, Abcam), anti-Bax antibody (1:1000, Abcam), anti-Cleaved Caspase-3 antibody (1:500, Abcam), anti-KDM2A antibody (1:1000, Abcam), anti-vascular endothelial growth factor (VEGF) antibody (1:1000, Abcam), and anti-GAPDH antibodies (1:2000, Abcam). On the second day, the membranes were cultured with antibody horseradish peroxidase-conjugated secondary antibody (1:2000, Santa Cruz) at room temperature for 1 hour. The blots were measured by enhanced chemiluminescence detection system (Roche Molecular Biochemicals).

Dual-Luciferase reporter (DLR) assay: The KDM2A 3'UTR fragment containing wild-type (WT) or mutant (Mut) miR-134-5p putative binding region was amplified and inserted into pGL3-KDM2A-3'UTR plasmid (Invitrogen). Then, miR-134-5p mimic (or its NC mimic) and the plasmids were co-transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen). Forty-eight hours later, the Luciferase activity was determined using DLR assay system (Promega, USA).

Immunofluorescence (IF) staining: After fixing in 4% paraformaldehyde and embedding in paraffin, myocardial tissues were cut into thin slices with 5-μm thickness. The tissue sections were blocked in goat serum for 30 minutes and cultured with anti-CD31 antibody (1:100, Abcam, Cambridge, MA, UK) at 4°C overnight. Next day, the tis-
Knockdown of miR-134-5p inhibited H/R-induced cardiomyocyte apoptosis: In the experiments, we examined the effect of miR-134-5p on cardiomyocyte apoptosis. MiR-134-5p inhibitor was transfected into cardiomyocyte to reduce miR-134-5p expression. Compared to the control group, H/R treatment upregulated miR-134-5p expression, while miR-134-5p inhibitor reversed the upregulation (Figure 2A). In addition, miR-134-5p inhibitor inhibited the increase of LDH level in the cell culture medium induced by H/R treatment (Figure 2B). MTT assay suggested that cardiomyocyte viability was lower in H/R+NC inhibitor group than the control group, while the cell viability was enhanced after miR-134-5p inhibitor transfection (Figure 2C). Further, western blot results showed that H/R treatment downregulated Bcl2 level and upregulated Bax and Cleaved Caspase-3 levels, whereas the effect of H/R treatment was diminished by miR-134-5p inhibitor (Figure 2D). These data suggested that miR-134-5p regulated the effect of H/R treatment on cardiomyocyte growth.

**KDM2A was the target gene of miR-134-5p:** To investigate the mechanism of miR-134-5p in cardiomyocytes, we predicted target gene that could bind to miR-134-5p through TargetScan and found that miR-134-5p could bind to 3'-UTR of KDM2A (Figure 3A). DLR assay suggested that miR-134-5p overexpression dramatically reduced the Luciferase reporter activity of KDM2A 3'-UTR-WT (Figure 3B). Moreover, Figure 3C revealed that the protein level of KDM2A was increased in miR-134-5p inhibitor group compared to the NC inhibitor group. Taken together, these results indicated that miR-134-5p negatively regulated KDM2A expression.

**MiR-134-5p regulated VEGF level through KDM2A:** Angiogenesis is one of the key processes for repairing and remodeling after MI. Thus, we determined whether miR-134-5p could regulate VEGF. Cardiomyocytes were treated with miR-134-5p inhibitor and shKDM2A after H/R treatment. As expected, Figure 4 showed that knockdown of miR-134-5p upregulated the protein level of KDM2A and VEGF in cardiomyocytes compared to H/R+NC inhibitor+shNC group. However, the effect of miR-134-5p inhibitor was offset by shKDM2A. Altogether, these data demonstrated that the role of miR-134-5p on MI might relate to VEGF and KDM2A.

**Silencing of miR-134-5p promoted myocardial angiogenesis and inhibited myocardial apoptosis in MI mice:** To investigate the effect of miR-134-5p on MI, mice were established MI mice model and measured the LDH release and infarction area. In contrast to the sham group, MI group was measured by LDH release assay. MI group (n = 8) was measured by TTC staining assay. C: MiR-134-5p expression in infarction tissues was analyzed by qRT-PCR. Data were expressed as mean ± SD. **P < 0.01 versus sham.

**Results**

**MiR-134-5p was highly expressed in the infarcted area of MI mice:** Firstly, we established MI mice model and measured the LDH release and infarction area. In contrast to the sham group, LDH level was increased in the serum of MI mice (Figure 1A). Moreover, the infarction area (white part in Figure 1B) was higher in MI group than in the sham group (Figure 1B). These indicated that MI mice were successfully modeled. Then, miR-134-5p expression in infarction tissues was detected by qRT-PCR assay, and Figure 1C results showed that its expression was increased in infarction tissues was detected by qRT-PCR assay, and Figure 1C results showed that its expression was increased in the sham group compared to the MI group. These results revealed that miR-134-5p expression was enhanced in the infarcted area of MI mice, indicating that miR-134-5p was related to MI development.

**Knockdown of miR-134-5p inhibited H/R-induced cardiomyocyte apoptosis:** In the experiments, we examined the effect of miR-134-5p on cardiomyocyte apoptosis. MiR-134-5p inhibitor was transfected into cardiomyocyte to reduce miR-134-5p expression. Compared to the control group, H/R treatment upregulated miR-134-5p expression, while miR-134-5p inhibitor reversed the upregulation (Figure 2A). In addition, miR-134-5p inhibitor inhibited the increase of LDH level in the cell culture medium.
Figure 2. The effect of miR-134-5p changes on cardiomyocyte apoptosis. Cardiomyocytes received H/R treatment and were transfected with miR-134-5p inhibitor (inh). A: MiR-134-5p expression was examined by qRT-PCR. B: LDH level in the cell culture medium was detected using LDH release assay. C: The function of miR-134-5p on cell viability was analyzed by MTT assay. D: The protein expressions of Bcl2, Bax, and Cleaved Caspase-3 were examined by western blot. **P < 0.01 versus Con. #P < 0.05 or ##P < 0.01 versus H/R + NC inhibitor.

Figure 3. KDM2A was negatively regulated by miR-134-5p. A: The binding sites between miR-134-5p and 3'-UTR of KDM2A were predicted through TargetScan. B: DLR assay was used to explore the relationship between miR-134-5p and KDM2A. C: The influence of miR-134-5p on KDM2A protein level. **P < 0.01 versus NC mimic or NC inh.

was increased in MI+NC antagonor group in contrast to the sham group, while miR-134-5p antagonor reversed the effect (Figure 5A). IF staining showed that CD31 density in MI+miR-134-5p antagonor group was higher than in MI+NC antagonor group (Figure 5B). The number of capillaries in MI+miR-134-5p antagonor group was also higher than the MI+NC antagonor group (Figure 5B). In addition, silencing of miR-134-5p diminished the increase of serum LDH level and infarct area caused by MI (Figure 5C, D). Further, western blot proved that miR-134-5p antagonor abrogated the reduction of KDM2A, VEGF, and Bcl2 expressions as well as the enhancement of Bax expressions.
Figure 4. MiR-134-5p regulated VEGF level through KDM2A. Cardiomyocytes were divided into control group, H/R + NC inhibitor + shNC group, H/R + miR-134-5p inhibitor + shNC group, H/R + NC inhibitor + shKDM2A group, and H/R + miR-134-5p inhibitor + shKDM2A group. The protein expressions of KDM2A and VEGF were examined by western blot. **P < 0.01 versus Con. #P < 0.05 or ##P < 0.01 versus H/R + NC inhibitor + shNC group. @P < 0.05 or @@P < 0.01 versus H/R + miR-134-5p inhibitor + shNC group.

Figure 5. Silencing of miR-134-5p promoted myocardial angiogenesis and inhibited myocardial apoptosis in MI mice. Mice were treated with miR-134-5p antagonist or NC antagonist by intramyocardial injection. Twenty-four hours after MI operation, myocardial tissues were collected for the following experiments. A: MiR-134-5p expression in infarction tissues was examined by qRT-PCR. B: IF staining was used to measure the CD31 density, and the average number of capillaries was calculated. C: Serum LDH level was detected using LDH release assay. D: The infarct size was detected by performing TTC staining assay. E: The protein expressions of KDM2A, VEGF, Bcl2, Bax, and Cleaved Caspase-3 were examined by western blot. **P < 0.01 versus sham. #P < 0.05 or ##P < 0.01 versus MI + NC antagonist.

and Cleaved Caspase-3 expressions induced by MI (Figure 5E). The above data revealed that knockdown of miR-134-5p inhibited MI development in vivo.

Discussion

MI is one of the common diseases that endanger human health. After MI, ventricular remodeling and heart
failure are two important factors affecting patients’ long-term survival and quality of life. Under hypoxia and other environmental stimuli, myocardial apoptosis, and angiogenesis are important processes of ventricular remodeling and heart failure after MI. Therefore, myocardial apoptosis and angiogenesis interventions in the early stage of MI can significantly improve the prognosis of MI. In the current study, we focused on miR-134-5p. We found that miR-134-5p was strongly expressed in infarction tissues of MI mice, and silencing of miR-134-5p could inhibit H/R-induced cardiomyocyte apoptosis and promote myocardial angiogenesis. We also revealed that the role of miR-134-5p in MI was related to KDM2A.

MiRNAs are a group of 20-25 nt long noncoding RNAs. Studies have confirmed that upregulated miRNAs in some diseases can promote the development of the corresponding diseases and downregulated miRNAs may inhibit the development process. For instance, overexpression of miR-17-3p in mice could promote functional recovery after cardiac ischemia/reperfusion, and inhibition of miR-200c suppressed hypoxia-induced cardiomyocyte apoptosis by targeting GATA-4. In MI, upregulation of miR-24 inhibited cardiomyocyte apoptosis in post-MI through targeting BIM, and overexpression of miR-210 improved heart function repair in MI rodents via promoting cardiomyocyte proliferation and angiogenesis. As one kind of miRNAs, miR-134-5p belongs to chromosome 14q32 miRNA clusters. It has been reported that miR-134-5p is associated with the development of several diseases. Qin et al. revealed that miR-134-5p could inhibit non-small cell lung cancer migration and invasion via decreasing ITGB1. Ji and his colleagues showed that miR-134-5p alleviated neuropathic pain development by down-regulating Twist1. In MI, miR-134-5p was found to be significantly elevated, suggesting that it may be involved in MI occurrence. However, the role of miR-134-5p in MI is unclear. In this study, we found that miR-134-5p was associated with the occurrence of MI, and miR-134-5p could regulate myocardial apoptosis and angiogenesis after MI. This is the first time to reveal the regulatory role of miR-134-5p in MI. Hence, researching the mechanism of miR-134-5p in MI is of great significance.

In general, miRNAs exert the role in many diseases by binding to the 3'-UTR of target genes. Based on the bioinformatics analysis of the miRNA target database (TargetScan), we found that KDM2A was the target gene of miR-134-5p. KDM2A is a multi-domain protein, which was the first identified jumonji C histone demethylase. Studies have shown that KDM2A can participate in regulating multiple cellular functions, including cell proliferation, apoptosis, and cell cycle. For example, overexpression of KDM2A promoted the growth and motility of gastric cancer cells. Inhibition of KDM2A reduced the proliferation, migration, and invasion of glioblastoma cells. In addition, KDM2A can also participate in the regulation of angiogenesis. Chen and his colleagues revealed that KDM2A accelerated angiogenesis of breast cancer via increasing Jagged1. In the present study, we discovered that miR-134-5p could negatively regulate KDM2A protein expression, and knockdown of miR-134-5p could upregulate VEGF protein level by upregulating KDM2A. VEGF is a pro-angiogenic cytokine, which can promote angiogenesis in the heart after MI. Thus, our above findings demonstrated that miR-134-5p could regulate cardiomyocyte apoptosis and angiogenesis via KDM2A. Through in vivo experiments, we confirmed that in the MI+miR-134-5p antagonist group, the MI size was reduced and the CD31 density was increased, indicating that knockout of miR-134-5p was effective in improving cardiac performance after MI. However, due to time and funding problems, we did not verify whether knockdown of KDM2A in mice would reverse the role of miR-134-5p antagonist, which will be validated in subsequent experiments.

Collectively, this study demonstrated that silencing of miR-134-5p could suppress myocardial apoptosis and promote myocardial angiogenesis after MI, and this role was related to KDM2A. This is the first study to report the mechanism of miR-134-5p in MI. These findings proposed that miR-134-5p might provide new clues and therapeutic targets for gene therapy after MI.

Disclosure

Conflicts of interest: The authors declare that they have no competing interests, and all authors should confirm its accuracy.

Availability of data and materials: All data generated or analyzed during this study are included in this published article.

Authors’ contributions: XL and CWW conceived and designed the experiments, ZZZ and QJ analyzed and interpreted the results of the experiments, and XX performed the experiments.

Ethics approval and consent to participate: The animal use protocol listed below has been reviewed and approved by the Animal Ethical and Welfare Committee.

Patient consent for publication: Not applicable.

Informed consent: Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

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Supplemental File

Supplemental Table
Supplemental Figure
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