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KLF9 aggravates ischemic injury in cardiomyocytes through augmenting oxidative stress

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ABSTRACT

Cardiomyocyte injury caused by excessive oxidative stress underlies the pathogenesis of myocardial infarction (MI), a devastating disease leading to heart failure and death. The Krüppel-like factor 9 (KLF9) is a transcriptional factor that has recently been reported to regulate oxidative stress, however, whether it is associated with cardiomyocyte injury and MI is unknown. We found that KLF9 was upregulated in the heart from a rat MI model. In addition, KLF9 was also upregulated in cardiomyocytes exposed to ischemia in vitro, suggesting that KLF9 responds to MI-relevant stimuli. Moreover, KLF9 knockdown protected cardiomyocytes against ischemic injury. Mechanistically, KLF9 knockdown reduced reactive oxygen species (ROS) generation in ischemic cardiomyocytes through upregulating the antioxidant thioredoxin reductase 2 (Txnrd2), and more important, Txnrd2 silencing abrogated KLF9 knockdown-mediated cardioprotection in ischemic cardiomyocytes. Altogether, these results suggest that KLF9 aggravates ischemic injury in cardiomyocytes through undermining Txnrd2-mediated ROS clearance, which might offer KLF9 as a possible target in alleviating MI.

1. Introduction

The ischemic heart disease (IHD) is one of the most prevalent diseases and the leading causes of death in developed countries [1]. Myocardial infarction (MI), caused by decreased blood supply to the myocardium, increases the risk for developing heart failure and high mortality in IHD patients [2,3]. MI is a highly complex process and its pathophysiology remains poorly understood. To maintain the heart function and reduce MI-related mortality in IHD patients, deciphering the molecular mechanisms leading to MI is urgently needed.

It has long been recognized that IHD is associated with increased production of reactive oxygen species (ROS) [4]. Upon myocardial ischemia, the depleted cellular ATP leads to mitochondrial dysfunction and excessive ROS production, which in turn causes oxidative stress and energetic deficit and damage of pivotal macromolecules including nucleic acids, proteins and lipids, thereby accelerating the death of cardiomyocytes through programs of apoptosis and necrosis [5,6]. Conversely, some anti-oxidant agents, such as vitamins [7], edaravone [8] and coenzyme Q10 [9], have been shown to alleviate heart injury and improve the clinical outcomes of MI patients. Therefore, targeting the molecular mechanisms involved in ROS elevation represents a promising therapeutic opportunity against oxidative stress-induced tissue damage in MI. Whereas, the regulation of ROS production and oxidative stress during the pathogenesis of MI remains largely obscure.

The Krüppel-like factor 9 (KLF9), a differentiation-associated transcription factor [10], belongs to the Sp1 C2H2-type zinc finger family [11]. KLF9 participates in a broad range of processes such as neural development [12], B cell differentiation [13], proliferation [14], and apoptosis [15]. Recently, KLF9 has been linked with oxidative stress regulation as a downstream target of the NF-E2-related transcription factor 2 (Nrf2), a key transcription factor inducing the expression of antioxidant genes [16]. In addition, KLF9-dependent ROS was reported to affect melanoma progression [17]. However, to our best knowledge, whether KLF9 is associated with MI and regulates cardiomyocyte injury have not been explored. In this study, we investigated the regulation and function of KLF9 using a rat MI model and an in vitro model in

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which cardiomyocytes were stimulated with ischemia.

2. Materials and methods

2.1. Cell isolation, culture and in vitro ischemia

The primary rat neonatal ventricular cardiomyocytes (RNVCs) were isolated from 1- to- 3-day-old neonatal Sprague-Dawley rats through digestion and Percoll gradient as described previously [18,19]. The isolated RNVCs were cultured at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% bovine calf serum and 100 U/ml penicillin/streptomycin in a humidified atmosphere of 5% CO_2. After 24 h, medium was replaced with serum-free DMEM supplemented with 10 μg/ml transferrin and 10 μg/ml insulin [20]. RNVCs were normally maintained in DMEM for 3–4 days, and then deprived of serum overnight to synchronize the growth. For in vitro ischemia treatment, RNVCs were exposed to ischemia (1% O_2, 0.1% glucose, serum-free) for 2 h to 24 h. RNVCs incubated in serum-free DMEM supplemented with 0.45% glucose under normoxia were used as treatment controls.

2.2. Plasmids, infection and transfection

The rat KLF9 and Txnrd2 cDNAs were cloned downstream of the CMV promoter of a lentiviral expression vector pLV-SV40-puro (Cellomics Technology, LVR-1006). The empty vector was used as a control. The lentiviral infection was performed according to protocols as described previously [21]. The siRNAs targeting negative control (siCtrl), KLF9 (siKLF9 #1, siKLF9 #2) or Txnrd2 (siTxnrd2) were synthesized by GenePharm (Shanghai, China). The transfection of siRNAs into RNVCs was performed using the Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

2.3. Animals and myocardial ischemia model

The animal model of myocardial ischemia (MI) was developed using 3- to- 4-month-old male Sprague-Dawley rats (SIPPR-BK Laboratory Animal Co. Ltd., Shanghai, China) as previously described [22]. In brief, Rats were anesthetized with intraperitoneal injection of 60 mg/kg sodium pentobarbitone. A left thoracotomy was carried out to expose the heart, and MI was developed by permanent ligation of the left anterior descending coronary artery (LAD) with a 6-0 prolene suture for 3 h to 72 h. Sham-operated rats without LAD ligation were used as surgical controls. All animal experiments were conducted in accordance with the Guide for Care and Use of Laboratory Animals approved by Guangdong Provincial Hospital of Integrated Traditional Chinese and Western Medicine.

2.4. Determination of ischemic injury

The ischemic injury was determined by the lactate dehydrogenase (LDH) activity, cell nonviability and apoptosis of RNVCs. LDH activity in culture medium and total cell lysates was determined using the LDH Cytotoxicity Assay Kit (Beyotime, C0017) according to the manufacturer's instructions. The LDH release was calculated as the proportion of LDH activity in culture medium among that in total cell lysates. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazonium bromide (MTT) assay [23]. Cell viability was calculated as percentage of control treatment. Apoptosis was detected by the TUNEL staining assay kit (Roche, 11684795910) according to the manufacturer's instructions. RNVCs were counterstained with DAPI to visualize the nuclei. Apoptosis was calculated as the proportion of TUNEL positive cells among the total cell number. Each treatment was performed in 5 replicates.

2.5. Western blotting analysis

The heart and cell samples were lysed using the RIPA lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100) containing a protease inhibitor cocktail tablet (Roche). The extracted protein samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with nonfat dry milk and probed with anti-KLF9 (Abcam, ab227920) antibody and anti-TXNRD2 (Abclonal, A5490) antibody, followed by incubation with the peroxidase-conjugated secondary antibody. Protein bands were detected by ECL chemiluminescence (Santa Cruz, sc-2048) and analyzed by ImageJ software.

2.6. Quantitative real-rime PCR

RNA from the heart and cell samples was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. The reverse transcriptase reaction was conducted using a SuperScript First-strand Synthesis System (Invitrogen) to synthesize complimentary cDNAs. Quantitative real-rime PCR (qRT-PCR) analysis was performed using an ABI Prism 7500 system (Applied Biosystems). Primers were purchased from RiboBio Co., Ltd. (Guangzhou, China).

2.7. ChIP assay

The ChIP assay was performed as described in a previous study [16]. The specific primers for amplifying the promotor region of the rat Txnrd2 are as the follows: Forward, 5′-CCATCCCTGAGCCCTGTAAC-3′; reverse, 5′-CCTCGGGGTCAGACCTGT-3′.

2.8. ROS measurement

RNVCs were collected and resuspended in Dulbecco's-PBS solution supplemented with 5 μg/ml of CM-H2DFCDA (Invitrogen, C6827) according to the manufacturer's instructions. RNVCs were incubated for 30 min with gentle shaking at 37 °C in the dark, and then centrifuged at 3000 rpm for 5 min to obtain cell pellets. The fluorescence (Ex/Em: ~492–495/517–527 nm) was analyzed by flow cytometry using the LSR IIA (BD Biosciences). The mean fluorescence intensity of at least 10,000 cells was analyzed by Flowjo software (Tree Star Inc., Ashland, USA).

2.9. Statistical analysis

All data are presented as the mean ± SD. Comparisons were performed using one-way ANOVA followed by post hoc Scheffe test with SPSS 18.0 (SPSS Inc., Chicago, IL, USA). A two-tailed p value < 0.05 was considered statistically significant for all analyses.

3. Results

3.1. KLF9 is elevated in experimental MI hearts

Although KLF9 was reported to be induced upon oxidative stress [16], a pathogenic condition tightly associated with myocardial infarction (MI) [6], whether KLF9 expression is directly responsive to MI is uncertain. In order to address this issue and explore whether KLF9 plays a functional role in MI, we initially examined its expression pattern in rat myocardium following experimental ischemia developed by ligating the left anterior descending (LAD) artery permanently [24]. Quantitative real-rime PCR (qRT-PCR) analysis showed that compared with that of non-infarcted counterparts and sham control, the mRNA level of KLF9 was gradually elevated in infarcted rat myocardium induced by LAD ligation for 3 h to 72 h (Fig. 1A). Likewise, similar trend was observed in protein level of KLF9 in rat myocardium measured by Western blotting analysis (Fig. 1B–C). These animal studies indicate

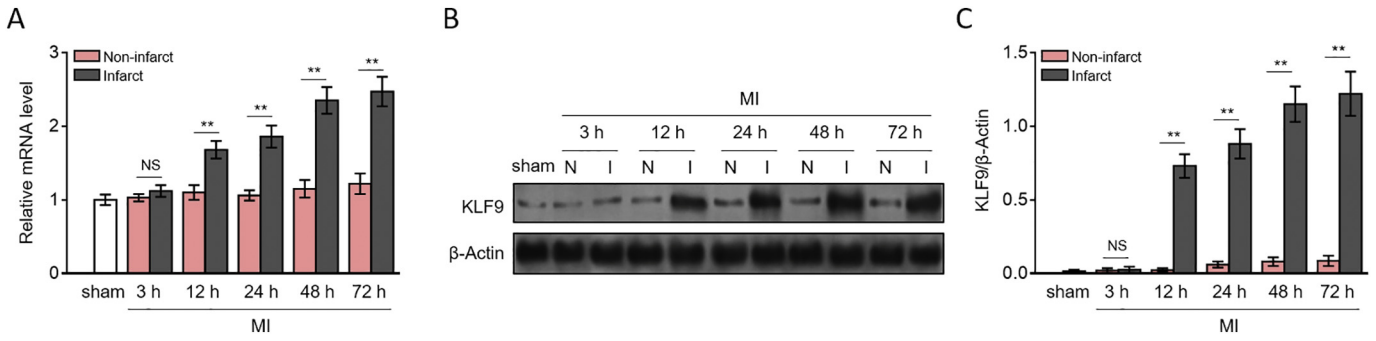

Fig. 1. KLF9 is upregulated in the heart from mouse MI model.
(A–C) The Sprague-Dawley rats were subjected to a permanent ligation of the left anterior descending (LAD) coronary artery for 3 h to 72 h as indicated (n = 8). Rats received sham surgery were used as controls (n = 4). The hearts were resected and the infarcted and non-infarcted myocardium were isolated and homogenized for following analyses. (A) The mRNA level of KLF9 was determined by qRT-PCR analysis. β-Actin was used as a reference control. (B–C) The protein level of KLF9 was determined by Western blotting analysis. β-Actin was used as a loading control. The blot images are representative of each group (B) and the quantification analysis of KLF9/β-Actin (C) is shown. N, non-infarct; I, infarct. All data are mean ± SD from each group and expressed as relative to that of sham control. One-way ANOVA followed by post hoc Scheffe test. **, $p < 0.01$; NS, not significant.

that KLF9 is upregulated in ischemic hearts in response to MI, and also imply that KLF9 may be associated with MI pathogenesis.

3.2. KLF9 is upregulated in ischemic cardiomyocytes in vitro

We then asked whether KLF9 could also be induced in cardiomyocytes under ischemia conditions in vitro. To test this possibility, we isolated the primary rat neonatal ventricular cardiomyocytes (RNVCs) and exposed them to simulative ischemia (1% O_2, 0.1% glucose, serum-free) under in vitro culture [25]. Notably, we found that similar to the findings observed in infarcted rat myocardium (Fig. 1), the mRNA level of KLF9 was increased in RNVCs stimulated with ischemia in a time-dependent manner, as compared with that under normoxia culture (Fig. 2A). Moreover, the protein level of KLF9 was also upregulated in ischemic RNVCs (Fig. 2B–C), further supporting that KLF9 could be an ischemia-responsive protein in cardiomyocytes. Next, we evaluated whether the upregulation of KLF9 occurs posttranscriptionally. As shown by Fig. 2D–E, actinomycin D, a transcription inhibitor, completely abrogated the ischemia-induced increase of KLF9 protein level, suggesting that the regulation of KLF9 does not occur post-transcriptionally under this condition.

3.3. KLF9 promotes ischemic injury in cardiomyocytes

The expression induction of KLF9 exposed to ischemia led us to ask whether it plays a role in ischemic injury in cardiomyocytes. To test this idea, we applied small interfering RNA (siRNA) technique to knock down the expression of KLF9 in RNVCs. Two different sets of siRNAs (siKLF9 #1 and siKLF9 #2) were transfected into RNVCs stimulated with or without ischemia (Fig. 3A), and the knockdown efficacy was evaluated by Western blotting analysis. As shown in Fig. 3B, the ischemia-induced expression of KLF9 in RNVCs was markedly decreased by siKLF9 #1 and siKLF9 #2, with siKLF9 #1 showing more robust effect. Next, we detected the lactate dehydrogenase (LDH) release of RNVCs, one commonly used indicator of cardiomyocyte injury [26]. As expected, the LDH release was increased in RNVCs stimulated with ischemia (Fig. 3C), indicating that ischemia indeed induces injury in RNVCs. Noticeably, the LDH release was attenuated in RNVCs transfected with both siKLF9 #1 and siKLF9 #2 (Fig. 3C), with siKLF9 #1 transfection displaying more profound reduction, which coincides with its better knockdown efficacy (Fig. 3B). These data suggest that KLF9 deficiency reduces cardiomyocyte injury induced by ischemia. Additionally, consistent with these, KLF9 deficiency via siRNA transfection significantly recovered the decreased cell viability of ischemia-treated RNVCs (Fig. 3D). Ischemia induces apoptosis in cardiomyocytes [27]. In agreement with KLF9 deficiency-increased cell viability of ischemic RNVCs, ischemia-induced apoptosis in RNVCs was also attenuated when KLF9 was knocked down (Fig. 3E). Hence, these results show that KLF9 deficiency alleviates ischemia-induced cardiomyocyte injury under an in vitro condition.

To confirm the role of KLF9 in ischemia-induced cardiomyocyte injury, we introduced its ectopic expression in RNVCs through lentivirus infection-mediated overexpression (Fig. 3F). The overexpression of KLF9 in RNVCs was first validated by Western blotting analysis (Fig. 3G). Just contrary to the results obtained from KLF9 knockdown (Fig. 3B–E), KLF9 overexpression resulted in a significant increase in LDH release (Fig. 3H), decreased cell viability (Fig. 3I), as well as enhanced cell apoptosis (Fig. 3J) in RNVCs stimulated with ischemia. Hence, these lines of evidence together demonstrate that KLF9 promotes ischemic injury in cardiomyocytes in vitro.

3.4. KLF9 elevates ROS level in ischemic cardiomyocytes through downregulating Txnrd2 expression

It has been shown that KLF9 is able to cause an increase in reactive oxygen species (ROS) in cultured cells and mouse tissues [16]. Besides, the elevated oxidative stress due to excessive production of ROS leads to cardiomyocyte death and MI-induced cardiac injury ([6,28,29]. Therefore, to elucidate how KLF9 exaggerates ischemic injury in cardiomyocytes, we subsequently focused on investigating whether and how KLF9 elevates ROS level in ischemia-stimulated cardiomyocytes. Consistent with previous reports [29,30], FACS analysis, combined with staining of a fluorescent probe CM-H2DFCDA [31], showed that ischemia treatment increased ROS level in RNVCs, indicating an elevated oxidative stress under this inferior culture condition (Fig. 4A). Remarkably, the elevated ROS level in ischemia-stimulated cardiomyocytes was diminished when KLF9 was knocked down by the transfection of siKLF9 #1 and siKLF9 #2 (Fig. 4A), suggesting that KLF9 may function to increase the production of ROS in cardiomyocytes in response to ischemia. This concept was further supported by the observation that KLF9 overexpression drastically increased ROS level in ischemia-stimulated RNVCs (Fig. 4B).

The ROS level in mammalian cells is tightly controlled by the antioxidant defense systems, including the family of thioredoxin reductases (Txnrd) [32]. Intriguingly, Txnrd2, an enzyme involved in ROS clearance, was previously identified as a target of KLF9 [16]. We wonder whether KLF9 increases ROS level in ischemia-stimulated RNVCs by regulating Txnrd2. As it turned out that KLF9 knockdown increased the expression of Txnrd2 (Fig. 4C), and by contrast, KLF9 overexpression decreased Txnrd2 expression in RNVCs (Fig. 4D), which

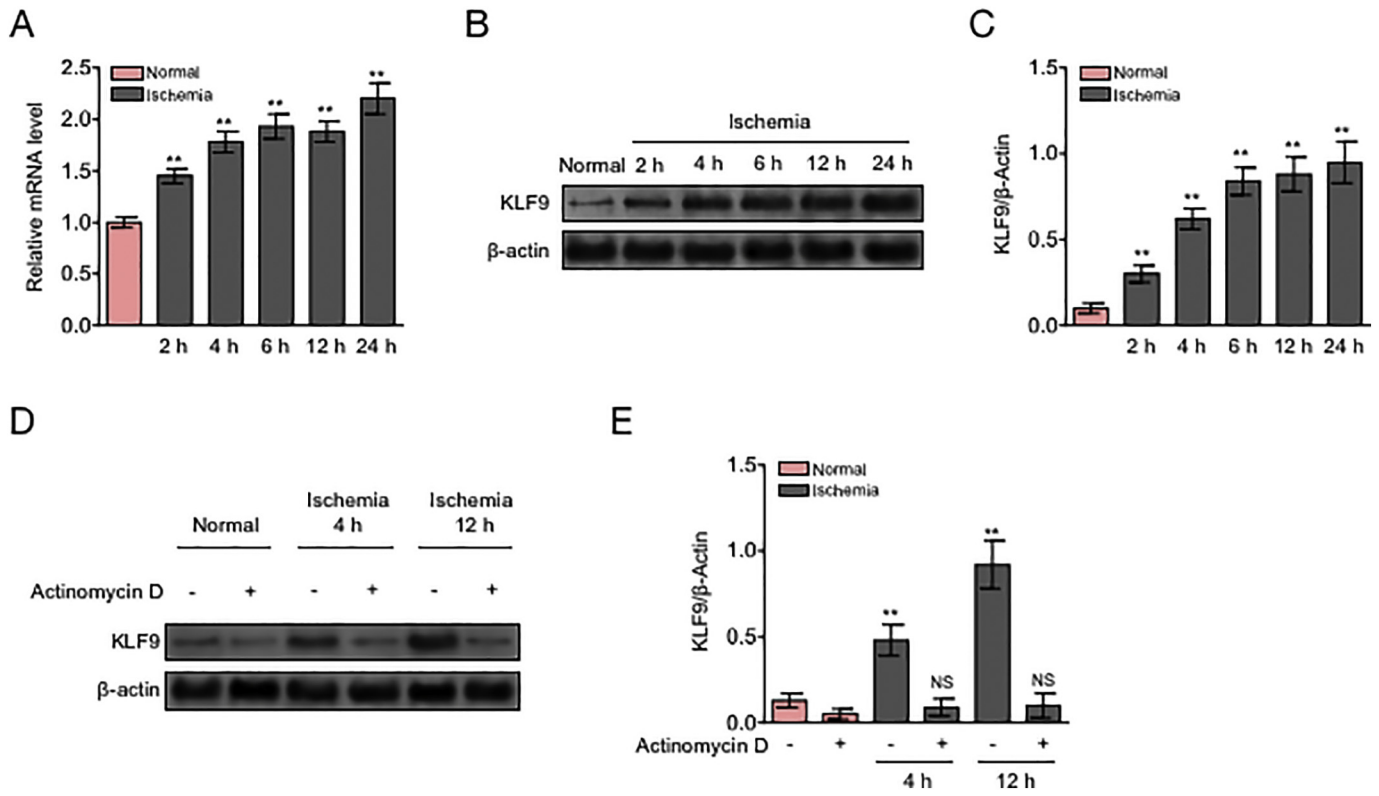

Fig. 2. KLF9 is upregulated in cardiomyocytes exposed to ischemia in vitro.
(A–C) The primary rat neonatal ventricular cardiomyocytes (RNVCs) were isolated and cultured under a normal condition or exposed to ischemia (1% O_2, 0.1% glucose, serum-free) for 2 h to 24 h as indicated. RNVCs were harvested and homogenized for following analyses. (A) The mRNA level of KLF9 was determined by qRT-PCR analysis. β-Actin was used as a reference control. (B–C) The protein level of KLF9 was determined by Western blotting analysis. β-Actin was used as a loading control. The blot images are representative of each group (B) and the quantification analysis of KLF9/β-Actin (C) is shown. (D–E) RNVCs were pretreated with or without 5 mg/ml actinomycin D for 1 h, followed by incubation with a normal condition or exposed to ischemia for 4 or 12 h. The protein level of KLF9 was determined by Western blotting analysis. The representative images (D) and the quantification analysis of KLF9/β-Actin (E) are depicted. All data are mean ± SD from each group and expressed as relative to that of normal control (n = 3). One-way ANOVA followed by post hoc Scheffe test. **, $p < 0.01$; NS, not significant versus the normal.

indicate a negative regulation of Txnrd2 by KLF9 under the ischemia condition. The chromatin immunoprecipitation (ChIP) assay showed that KLF9 bound to Txnrd2 promoter and their binding was enhanced upon ischemia (Fig. 4E). To clarify whether Txnrd2 contributes to KLF9-modulated ROS level in ischemia-stimulated RNVCs, we knocked down Txnrd2 through siRNA transfection in RNVCs depleted of KLF9 (Fig. 4F). Evidently, we found that the decreased ROS level caused by KLF9 deficiency was totally recovered by Txnrd2 knockdown (Fig. 4G), suggesting that the induction of Txnrd2 mediates the antioxidant effect of KLF9 deficiency. To consolidate this mechanism, Txnrd2 was overexpressed in RNVCs which were concomitantly enforced to express KLF9. The downregulation of Txnrd2 by enforced KLF9 expression was reversed by lentivirus infection-mediated Txnrd2 overexpression (Fig. 4H). Moreover, keeping in line with the restored Txnrd2, the KLF9-induced ROS elevation in ischemia-stimulated RNVCs was completely abolished (Fig. 4I). Thus, these data collectively demonstrate that KLF9 increases ROS level in ischemic cardiomyocytes by suppressing Txnrd2 expression via direct binding.

3.5. Txnrd2 mediates protective role of KLF9 knockdown against ischemic injury in cardiomyocytes

To establish whether Txnrd2 is the causal link between KLF9-modulated ROS level and ischemic injury in cardiomyocytes, we checked the effect of Txnrd2 manipulation on ischemia-stimulated RNVCs. We found that the KLF9 deficiency-ameliorated ischemic injury in RNVCs, including LDH release (Fig. 5A), reduced cell viability (Fig. 5B), and cell apoptosis (Fig. 5C), were all drastically diminished when Txnrd2 was also knocked down. Furthermore, in concert with these results, the promoted ischemic injury in RNVCs by KLF9 overexpression was substantially attenuated by restored expression of Txnrd2 (Fig. 5D–F). Combining with the functional role of Txnrd2 in mediating KLF9-modulated ROS level (Fig. 4), these findings suggest that the detrimental effect of KLF9 on ischemic injury in cardiomyocytes relies on its negative regulation of Txnrd2, which is essential for the antioxidant defense against ROS generation.

4. Discussion

The myocardial oxidative stress caused by elevated ROS production during the process of MI triggers tissue damage and cardiomyocyte death through apoptosis, leading to exacerbated myocardial ischemia injury [33–35]. In MI patients, some oxidative stress markers, such as C-reactive protein [36] and thiol/disulphide [37], are also increased. Further, in transgenic mice overexpressed with superoxide dismutase (SOD), an antioxidant protein, the infarct size is markedly minimized [38,39]. Therefore, strategies aimed at scavenging ROS accumulation have long been considered to be appropriate options to alleviate oxidative stress-induced myocardial ischemia injury [7,40]. However, the molecule mechanisms that regulate ROS level in the pathogenesis of MI are still not fully uncovered. In the current study, we found that the transcriptional factor KLF9 was induced in infarcted hearts from rats with experimental MI as well as in cardiomyocytes exposed to ischemia condition in vitro. We subsequently demonstrated that KLF9 promotes

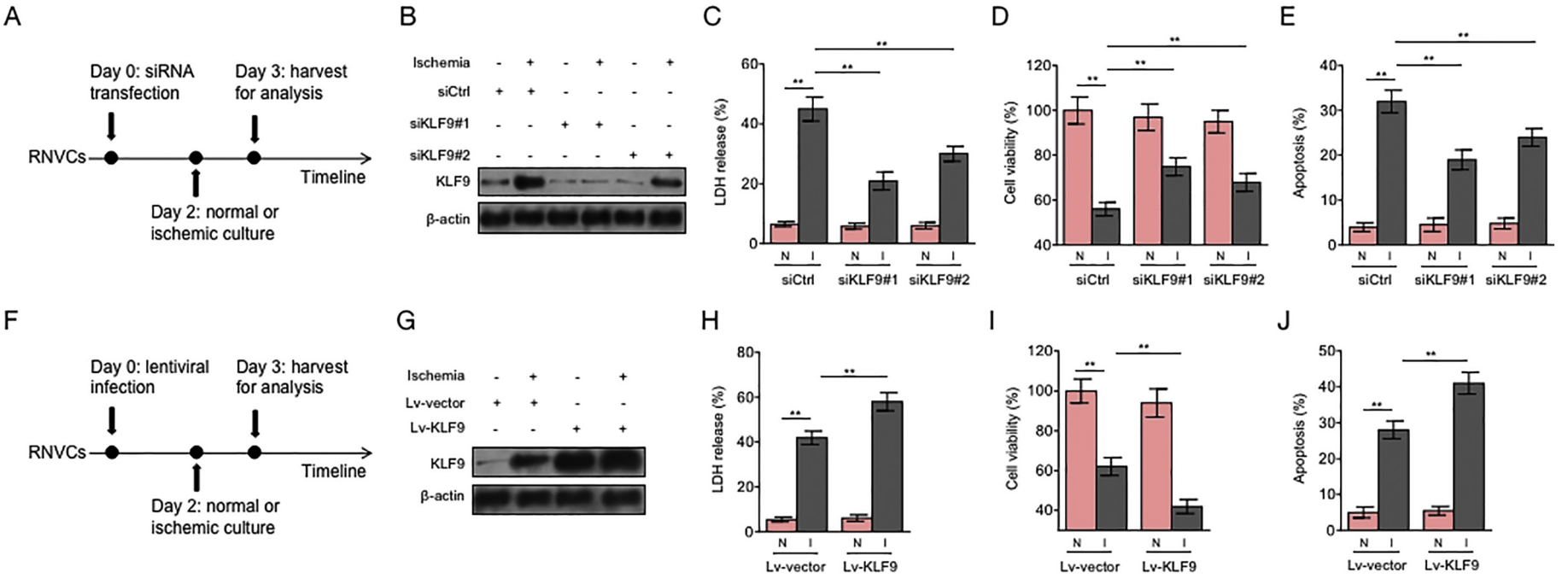

Fig. 3. KLF9 promotes cardiomyocyte ischemic injury.

(A–E) RNVCs were transfected with negative control siRNA (siCtrl) or two different sets of siRNAs targeting KLF9 (siKLF9 #1, siKLF9 #2). After 48 h, RNVCs were cultured under a normal condition or exposed to ischemia for 24 h (A). (B) RNVCs were homogenized and the protein level of KLF9 was determined by Western blotting analysis. β-Actin was used as a loading control. The blot images are representative of each group (n = 3). N, normal; I, ischemia. (C) The lactate dehydrogenase (LDH) activity in the media was measured and expressed as a percentage within the total LDH activity of cell lysates (n = 5). (D) The cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazonium bromide (MTT) assay. Data are expressed as relative to that of siCtrl group (n = 5). (E) The cell apoptosis was evaluated by TUNEL staining. Data are expressed as the percentage of TUNEL positive apoptotic cells within the total cell number (n = 5). (F–J) RNVCs were infected with lentivirus expressing empty vector (Lv-vec) or KLF9 (Lv-KLF9). After 48 h, RNVCs were cultured under a normal condition or exposed to ischemia for 24 h (F). The protein level of KLF9 (G), LDH release (H), cell viability (I), and cell apoptosis (J) were analyzed as in (B–E). All data are mean ± SD. One-way ANOVA followed by post hoc Scheffe test. **, $p < 0.01$.

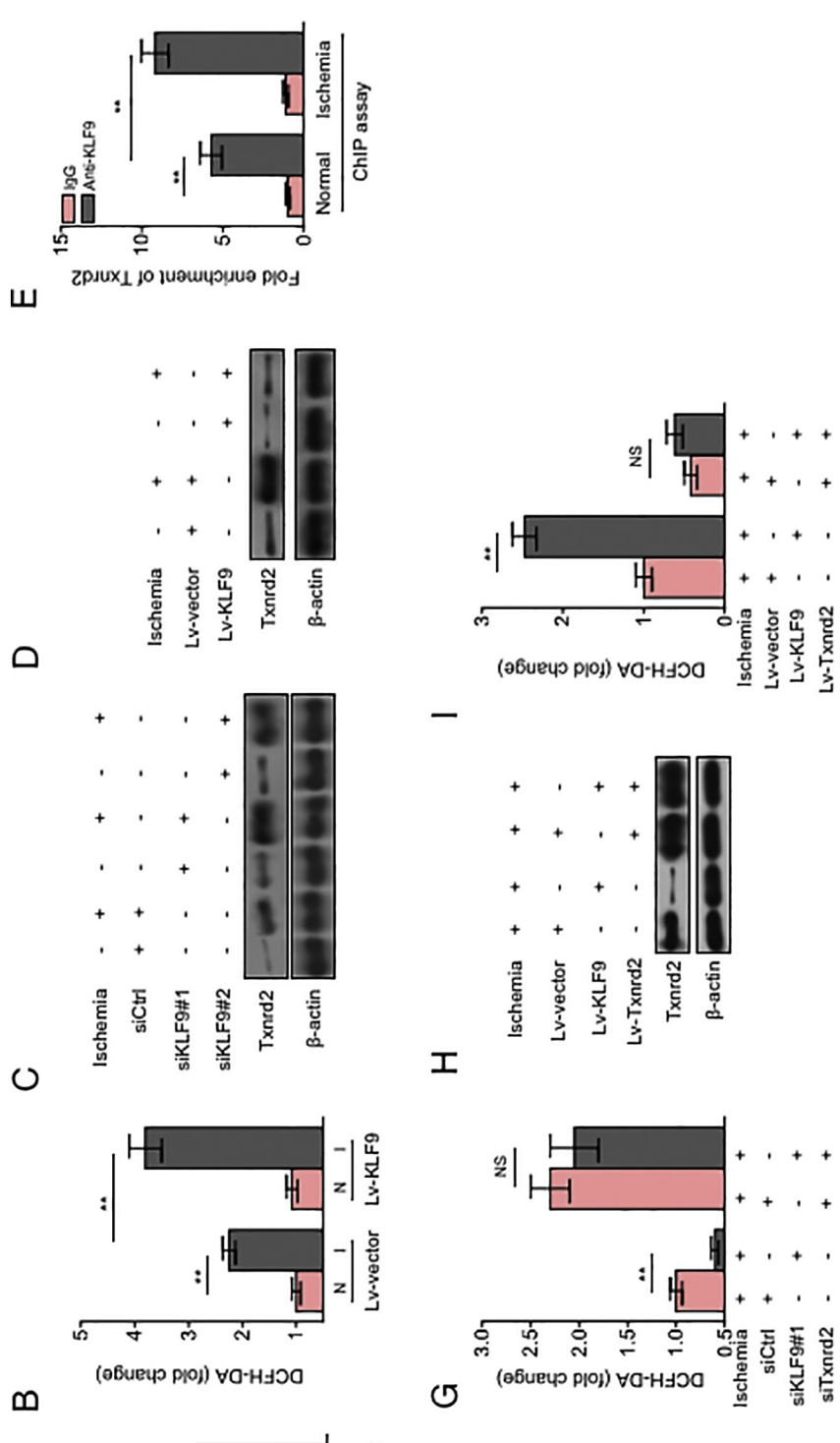

Fig. 4. KLF9 increases ROS generation in ischemic cardiomyocytes through downregulating Txnrd2.
(A) RNVCs were transfected with siCtrl, siKLF9 #1 or siKLF9 #2. After 48 h, RNVCs were cultured under a normal condition or exposed to ischemia for 24 h as indicated. The intracellular ROS level was measured by DCFH-DA staining combined with flow cytometry analysis. Data are expressed as fold change relative to siCtrl group (n = 4). (B) RNVCs were infected with Lv-vec or Lv-KLF9. After 48 h, RNVCs were cultured under a normal condition or exposed to ischemia for 24 h as indicated. The intracellular ROS level was measured as in (A) (n = 4). (C–D) RNVCs were treated as in (A-B). The protein level of Txnrd2 was determined by Western blotting analysis. β-Actin was used as a loading control. The blot images are representative of each group (n = 3). (E) ChIP assay with anti-KLF9 using lysates from RNVCs cultured under a normal or ischemic condition. IgG antibody was used as a control. DNA samples were analyzed by qRT-PCR with primers amplifying promoter region of the rat Txnrd2 gene. The fold enrichment of Txnrd2 relative to IgG is expressed. (F–G) RNVCs were transfected with siCtrl or siKLF9 #1 in combination with or without siTxnrd2. After 48 h, RNVCs were exposed to ischemia for 24 h as indicated. (F) The protein level of Txnrd2 was determined by Western blotting analysis. β-Actin was used as a loading control. The blot images are representative of each group (n = 3). (G) The intracellular ROS level was measured by DCFH-DA staining combined with flow cytometry analysis. Data are expressed as fold change relative to siCtrl group (n = 4). (H–I) RNVCs were infected with Lv-vec or Lv-KLF9 in combination with or without Txnrd2 overexpression. After 48 h, RNVCs were exposed to ischemia for 24 h as indicated. The protein level of Txnrd2 (H) and intracellular ROS level (I) were analyzed as in (F–G). All data are mean ± SD. One-way ANOVA followed by post hoc Scheffe test. **, $p < 0.01$; NS, not significant.

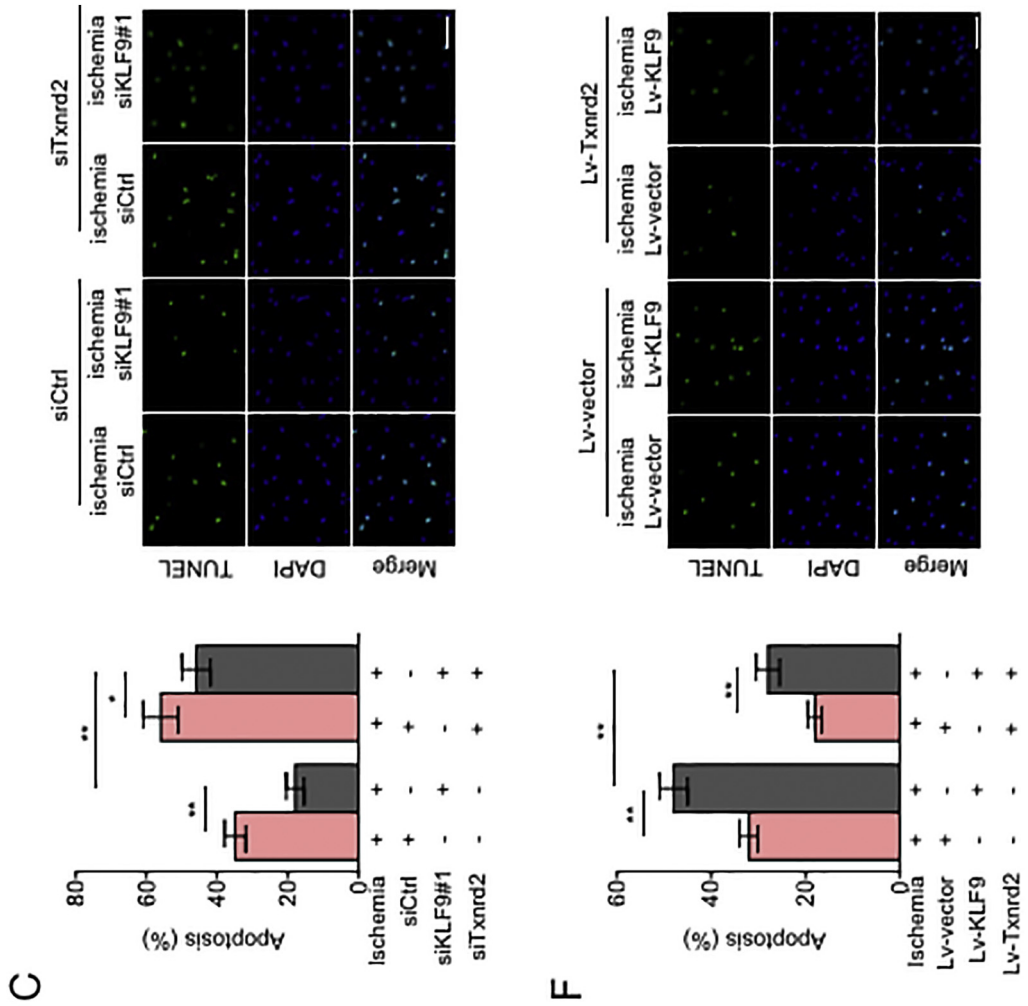

Fig. 5. Txnrd2 mediates protection of KLF9 knockdown against cardiomyocyte ischemic injury.
(A–C) RNVCs were transfected with siCtrl or siKLF9 #1 in combination with or without siTxnrd2. After 48 h, RNVCs were exposed to ischemia for 24 h as indicated. (A) The LDH activity in the media was measured and expressed as a percentage within the total LDH activity of cell lysates (n = 5). (B) The cell viability was measured by MTT assay. Percentage of viable cells is shown (n = 5). (C) Cell apoptosis was evaluated by TUNEL staining. Data are expressed as the percentage of TUNEL positive apoptotic cells within the total cell number (n = 5). The representative fluorescent images are shown right. (D–F) RNVCs were infected with Lv-vec or Lv-KLF9 in combination with or without Txnrd2 overexpression. After 48 h, RNVCs were exposed to ischemia for 24 h as indicated. The LDH release (D), cell viability (E), and cell apoptosis (F) were analyzed as in (A–C). All data are mean ± SD. One-way ANOVA followed by post hoc Scheffe test. *, $p < 0.05$; **, $p < 0.01$; NS, not significant. Scale bar = 50 μm.

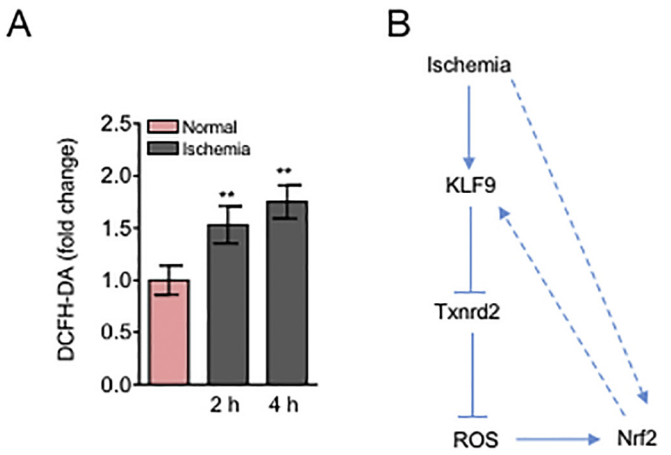

Fig. 6. ROS and KLF9 may form an auto-amplifying loop upon ischemia. (A) RNVCs were cultured under a normal condition or exposed to ischemia for 2 or 4 h. The intracellular ROS level was measured by DCFH-DA staining combined with flow cytometry analysis. Data are expressed as fold change relative to normal group (n = 4). (B) A brief schematic model of this study, in which an auto-amplifying loop may exist between ROS and KLF9 upon ischemia. Data are mean ± SD. One-way ANOVA followed by post hoc Scheffe test. **, $p < 0.01$ versus the normal.

cardiomyocyte ischemic injury through increasing intracellular ROS level via the downregulation of Txnrd2, a critical anti-oxidant enzyme. Thus, our study may identify KLF9 as a new positive regulator of ROS level in MI pathogenesis, which might be exploited as a potential therapeutic target for reducing myocardial ROS accumulation and alleviating oxidative stress-exacerbated MI injury.

We show that both the mRNA level and protein level of KLF9 are upregulated in ischemic rat hearts and cardiomyocytes, which suggest that KLF9 may be an ischemia-responsive transcriptional factor. Coincidely, in an integrated analysis of publicly available Gene Expression Omnibus datasets, KLF9 was identified to be upregulated in MI patients [41]. Interestingly, it has been reported that KLF4 is also upregulated in ischemic myocardium [42]. We suspect that some other Krüppel-like transcription factors (KLFs) are possibly involved in MI as well. At present, the factors that regulate KLF9 transcription are scarcely characterized. In human endometrial epithelium, KLF9 expression is inhibited by HOXA10 [43]. In addition, in purified oligodendrocyte precursor cells, KLF9 was found to be induced by the thyroid hormone-driven signaling cascade [44]. Possibly more relevant to our study, since *KLF9* contains several response elements for binding Nrf2, it can be transcriptionally induced by Nrf2 upon the intracellular ROS exceeding a critical threshold [16]. As known, the activation of Nrf2 reduces ischemic injury in multiple organs through ameliorating oxidative stress, such as brain [45], kidney [46], and heart [47]. The critical role of Nrf2 in protecting against MI injury can be well demonstrated by a rapid progression to heart failure following MI in mice lacking Nrf2 [48]. Moreover, Nrf2 reduces ischemic injury in cardiomyocytes in vitro [49,50]. Therefore, we suppose it is very likely that Nrf2 contributes to expression induction of KLF9 in ischemic rat hearts and cardiomyocytes. As a support for this presumption, we found that the ROS level was significantly elevated in RNVCs as early as 2 h following ischemia (Fig. 6A), which is synchronized with the upregulation of KLF9 (Fig. 2), suggesting that KLF9 could be, in turn, activated by ROS through Nrf2, thus forming an auto-amplifying loop between ROS and KLF9 upon ischemia (Fig. 6B). Yet, except for Nrf2, the possibility that other factors may also be involved in KLF9 regulation cannot be ruled out either. Further studies are required to confirm whether Nrf2 accounts for KLF9 upregulation and discover other possible upstream regulators of KLF9 under ischemia exposure. On the other hand, it should be noted that we merely performed experiments using Sprague-Dawley rat MI model and RNVCs stimulated with in vitro ischemia. Whether KLF9 displays expression induction in other MI models is uncertain. In the future, it is of interest and clinical significance to examine whether KLF9 is induced in human MI samples, which could provide a useful hint to establish possible clinical relevance between KLF9 and MI pathogenesis.

In the latest studies, some members of KLFs have been shown to play a role during ischemic condition. For example, the genetic deletion of KLF11 aggravates ischemic brain injury [51]. In addition, KLF2 and KLF4 protect endothelial cells from ischemic stroke-induced apoptosis [52,53]. KLFs also participate in some cardiac activities. KLF13 protects adult cardiomyocytes from DNA damage and death [54]. KLF15, KLF11 and KLF6 were found to regulate cardiac hypertrophy and fibrosis [55–57]. KLF9 regulates drug-induced apoptosis in multiple myeloma cells [15], and also controls ROS-dependent catastrophic cell death in medullary thyroid cancer [58]. In this study, we reveal that KLF9 promotes ischemic injury in cardiomyocytes, as demonstrated by increased LDH release, decreased cell viability, and enhanced cell apoptosis. The ischemia-induced apoptosis of cardiomyocytes is a critical event linked with MI injury and heart failure [59,60]. Based on the presented evidence, we estimate that targeting KLF9 might be useful to maintain the viability of cardiomyocytes and reduce their loss during MI.

The Txnrd2 polymorphism has been considered as a risk factor for MI in patients with type 2 diabetes mellitus [61]. It's also reported that Txnrd2 reduces mitochondrial ROS and is essential for early post-ischemic myocardial protection [62]. In a previous study, KLF9 was proved to suppress Txnrd2 so as to amplify oxidative stress, which underlies the bleomycin-induced pulmonary fibrosis and lung injury in mice [16]. We demonstrate that KLF9 elevates ROS level in ischemic cardiomyocytes by downregulating Txnrd2 expression, and that Txnrd2 restoration not only diminishes KLF9-elevated ROS level, but also ameliorates KLF9-aggravated ischemic injury in cardiomyocytes. Thus, we relate the detrimental effect of KLF9 on cardiomyocyte ischemic injury to its negative regulation of Txnrd2-mediated ROS clearance, which provide another example the anti-oxidative enzyme Txnrd2 protects against ischemic injury. Due to the direct regulation of Txnrd2 by KLF9, it is possible that KLF9 may interfere with the anti-oxidative activity of Txnrd2 in other circumstances, such as cerebral and renal ischemic injury. More efforts would be required to investigate whether KLF9 plays a role in the pathogenesis of other oxidative stress-related diseases.

In summary, we report that KLF9 aggravates ischemic injury in cardiomyocytes through modulating Txnrd2-mediated antioxidant defense machinery, which may provide a molecular foundation for the therapeutic application of targeting KLF9/Txnrd2 axis in MI therapy.

Declaration of Competing Interest

None.

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