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KLF6 aggravates myocardial fibrosis by promoting mitochondrial division

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Abstract: The dysregulation of mitochondrial dynamics in cardiac fibroblasts (CFs) is closely linked to myocardial fibrosis, which can induce cardiac dysfunction and even lead to heart failure. As an essential multifunctional zinc-finger transcriptional factor of cardiovascular remodeling, the role of KLF6 mediating the link between mitochondrial fission and myocardial fibrosis remains unclear. Next, we want to explore whether the effect of KLF6 on mitochondrial fission might influence cardiac fibroblasts, we established a model of Transforming growth factor β1 (TGF-β1) and Isoprenaline (ISO)-induced myocardial fibrosis. Here, we found that KLF6 up-regulation in CFs is correlated with myocardial fibrosis. While knockdown of KLF6 suppresses mitochondrial fission and the Keap1/Nrf2 pathway molecules, which alleviates myocardial fibrosis induced by TGF-β1. Our findings not only clarified the regulation mechanism of mitochondrial fission by KLF6 but also provided a potential therapeutic target for cardiovascular disease.

Keywords: KLF6, myocardial fibrosis, mitochondrial fission, oxidative stress, Keap1/Nrf2 pathway.

INTRODUCTION

Mitochondria, as organelles, play crucial roles in various cellular processes (Chen W, *et al.*, 2023; Giacomello M, *et al*., 2020), including energy metabolism and the regulation of reactive oxygen species (ROS) production (Liu Y *et al.*, 2023; Andrieux P *et al*., 2021). These processes are tightly regulated by mitochondrial dynamics, which involve the continuous processes of fission and fusion. Fusion, the process by which mitochondria merge together, is mediated by proteins such as mitofusin 1 (MFN1) and mitofusin-2 (MFN2). These proteins accelerate the fusion of the mitochondrial outer membranes, allowing the exchange of contents and the maintenance of mitochondrial integrity. On the other hand, fission, the division of mitochondria into smaller units, is primarily regulated by dynamin-related protein 1 (Drp1). Drp1 is recruited from the cytoplasm to the mitochondrial outer membrane (Vasquez *et al*., 2016), where it constricts and divides the mitochondria.

The balance between fusion and fission is crucial for maintaining mitochondrial morphology, distribution and function within the cell. The dysregulation of mitochondrial dynamics has been associated with various pathological conditions, such as neurodegenerative diseases, cancer, and metabolic disorders. Mitochondria can maintain the balance of dynamics by continuously regulating fission and fusion (Adebayo *et al*., 2021; Rodrigues *et al*., 2020), changing the morphology and number of mitochondria to meet the metabolic needs of cells (Chan, 2020; Lin *et al.*, 2022). Mitochondrial homeostasis is disrupted when cells are subjected to metabolic or environmental stress and have been closely related to various diseases (Chen *et al*., 2023; Zacharioudakis *et al*., 2023), particularly cardiovascular diseases (Forte *et al*., 2021).

KLF6 is a multipotent zinc finger transcription factor (Li *et al.*, 2023; Syafruddin *et al.*, 2020; Chen *et al*., 2022). The N-terminal of KLF6 protein contains a domain related to transcriptional activation (Syafruddin *et al.*, 2020), which can participate in phosphorylation (Mertins *et al.*, 2013), acetylation (Li *et al.*, 2005) and other post-transcriptional regulation. Previous studies have reported that Ang II can specifically up-regulate the expression level of the KLF6 gene in mouse cardiomyocytes and increase KLF6 recruitment on the TSP4 promoter, thereby activating fibroblasts (Sawaki *et al.*, 2015). Knockdown of KLF6 in mice can alleviate the development of cardiac fibrosis compared with wild-type mice (Sawaki *et al.*, 2015).

However, the role of KLF6 in regulating mitochondrial dynamics is unclear and its precise molecular mechanisms need to be urgently investigated. Therefore, this study focused on whether and how KLF6 influences myocardial fibrosis by regulating mitochondrial dynamics. In our study, we interpreted that KLF6 accelerated mitochondrial fission. We explore its possible mechanism and found that Keap1/Nrf2 pathway could be regulated by KLF6 in cardiac fibroblasts. In addition, KLF6 was associated with

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myocardial fibrosis markers. These results revealed that KLF6 was a new regulator of mitochondrial dynamics and might be used as a new aim molecule for the treatment of heart disease.

MATERIALS AND METHODS

Animals

6-8-week-old male wild-type (WT) C57BL/6J mice (18-23 g) were purchased from the Liaoning Changsheng Biotechnology Co., Ltd, (License No. SCXK Liaoning 2020-0002). All procedures were approved by the Institutional Animal Care and Use Committee of Hebei University of Chinese Medicine authorized all processes (No. DWLL2020018). During the 7-day experimental period, all mice were kept at 21-27°C and given free food and water. Myocardial fibrosis models were made according to the literature previously reported (Wan *et al.*, 2018). In summary, mice were injected subcutaneously with ISO (5 mg/kg/day) for 7 days. Finally, mice were humanely killed, then their heart was collected for further experiment.

HE and masson staining

Initially, the hearts were fixed using 4% paraformaldehyde (Servicebio, G1101, Wuhan, China) for pathological examination. The fixed hearts were processed with paraffin and sectioned into slices 5μm slice. Then, we stained the slices with HE and Masson trichrome staining (Solarbio, G1120, Beijing, China). A light microscope was used to capture pictures (Leica, DM6B, Germany). The remainder was stored at −80°C.

Immunohistochemistry

The heart slides (5 μm) were de-paraffinized to water. Using sodium citrate buffer (PH=6.0) (Servicebio, Wuhan, China) to repair epitopes and then naturally cooled to room temperature. After blocking endogenous peroxidase, 5% goat serum (Zhongshan Golden Bridge Biotechnology, Beijing, China) was used to block the heart slides. The primary antibody KLF6 (Proteintech; 14716-1-AP, 1:100) incubated overnight at 4°C.

The second day, we using PBS to wash slices. We useing secondary antibody to incubate the slices at room temperature for half an hour. A diaminobenzidine kit (Zhongshan Golden Bridge Biotechnology, Beijing, China) was used to Immunohistochemical staining following the product manual. Hematoxylin staining was then performed to visualize the nuclei in the sections.

Cell culture

Human cardiac fibroblasts (HCFs) were obtained from Shanghai Honsun Biological Technology Co., Ltd (China). The high-glucose medium (Gibco, USA) replenished with 1% penicillin-streptomycin (Gibco, USA) and 10% fetal bovine serum was used to culture HCFs. The cells were cultured in a cell incubator at 37°C with 5% CO_2.

Cell transfection

The siKLF6 siRNA sequences were purchased from GenePharma (Shanghai, China). We used the Lipofectamine 3000 reagent (Invitrogen) to transfect the siRNAs. The sequences used are shown in table 1.

Table 1: siRNA sequences for siKLF6

siRNA	Sequence
siRNA 1-sense(3'-5')	GAAUCCGAACUGAAGAUAUTT
siRNA 1-antisense(5'-3')	AUAUCUUCAGUUCGGAUUCTT
siRNA 2-sense(3'-5')	GACAGCUCCGAGGAACUUUTT
siRNA 2-antisense(5'-3')	AAAGUUCCUCGGAGCUGUCTT
siRNA 3-sense(3'-5')	GCCAGGUGACAAGGGAAAUTT
siRNA 3-antisense(5'-3')	AUUUCCCUUGUCACCUGGCTT

Western blot

Proteins were incubated with primary antibody of KLF6 (Proteintech, 14716-1-AP, 1:1000), MFN1 (Proteintech, 13798-1-AP, 1:1000), MFN2 (Proteintech, 12186-1-AP, 1:1000), Drp1 (Proteintech, 12957-1-AP, 1:1000), Collagen Ⅰ (abcam, ab270993, 1:1000), Collagen Ⅲ (abcam, ab7778, 1:1000), α-SMA (abcam, 32575, 1:4000), Keap1 (Proteintech, 10503-2-AP, 1:1000), Nrf2 (Proteintech, 16396-1-1AP, 1:1000), GAPDH (Proteintech, 60004-1, 1:10000), incubated overnight at 4°C. The following day, the proteins were exposed to a secondary antibody at room temperature for 1.5 hours. Proteins bands were detected by ECL (NCM Biotech, Suzhou, China) and the image was captured by the chemiluminescence imager Omegalum W (USA).

Measurement of Mitochondrial reactive oxygen species

Using kit to conduct Mitochondrial reactive oxygen species staining according to instructions. Add 1 mL of 37°C pre-warmed probe working solution (10 μmol/L) (Beyotime, Shanghai, China) to the cells and incubate for 20 minutes at 37°C in the dark with 5% CO_2. Then, the above staining solution was replaced with a fresh medium. Finally, the cells were examined under a fluorescence microscope.

STATISTICAL ANALYSIS

All data were evaluated using GraphPad Prism 8 software (San Diego, CA) and the mean is shown as mean ± standard deviation (SD). The one-way analysis of variance (ANOVA) was used to determine statistical significance among groups. Multiple comparison testing was performed using the Bonferroni method. $P<0.05$ was judged as statistically significant.

RESULTS

Myocardial fibrosis induces upregulation of KLF6 expression and promotes mitochondrial fission

To illustrate the significant role of mitochondria dynamics in the pathological process of myocardial fibrosis, we established a myocardial fibrosis model induced by ISO. Then, we found that the ISO group significantly increased Collagen disorder and deposition of Collagen as shown by HE and Masson staining (fig. 1A-B). These results showed that the myocardial fibrosis model in mice was successfully built. Immunohistochemical stains of KLF6 showed that compared with the control group, KLF6 expression increased and mainly enriched in the nucleus in the ISO group (fig. 1C). Next, the Western blot confirmed that the ISO group significantly increased the expression of KLF6 and Drp1 protein. On the contrary, MFN1 and MFN2 significantly decreased (P<0.001) (fig. 1D-E). These results showed that myocardial fibrosis induced upregulation of KLF6 expression and promotes mitochondrial fission.

(A-B) Representative images of HE and Masson staining of left ventricular tissue in different groups. Scale bars=100 μm. (C) Immunohistochemical analysis of KLF6 protein expression in heart cross sections of different groups on day 7. Scale bars=50 μm. (D-E) Mice were treated with ISO, and the expression of KLF6, Drp1, MFN1, and MFN2 was analyzed by Western blot. The data are presented as the mean ± SD. Data are analyzed using one-way ANOVA followed by Tukey's post-hoc test. ***P<0.001 versus the corresponding control.

Down-regulation of KLF6 inhibits myocardial fibrosis induced by TGF-β1

To further clarify the relationship between KLF6 and myocardial fibrosis, we observed a significant reduction in KLF6 expression in cardiac fibroblasts (CFs) following the knockdown of KLF6 expression. CFs transfected with different fragments of KLF6 siRNA exhibited decreased KLF6 expression compared to controls (P<0.01, P< 0.001) (fig. 2A-B). Then, KLF6 was knocked down and treated with TGF-β1 in CFs. The Western blot revealed that TGF-β1-induced elevation of Collagen I, Collagen Ⅲ, and α-SMA were severely diminished (P<0.01, P<0.001) (fig. 2C-D). These results suggest that siKLF6 suppressed myocardial fibrosis induced by TGF-β1.

(A-B) CFs were transfected with si-KLF6 and the expression of KLF6 was analyzed by Western blot. (C-D) CFs were transfected with si-KLF6 and then treated or not with TGF-β1 (10 ng/mL) for 24h. The expression of Collagen I, Collagen III and α-SMA was analyzed by Western blot. The data are presented as the mean ± SD. Data are analyzed using one-way ANOVA followed by Tukey's post-hoc test. **P<0.01, ***P<0.001, ## P< 0.01, ###P<0.001 versus the corresponding control.

KLF6 induced mitochondrial dynamics disorder

The above-mentioned data illustrated that KLF6 was closely related to mitochondrial dynamics. In order to further explore the relationship between KLF6 and mitochondrial fission, we over expressed KLF6 expression in CFs.

The results demonstrated that the expression of KLF6 was elevated compared to the control group. (P<0.001) (fig. 3A-B). Next, we utilizing loss and gain-of-function experiments, we verified that over expression of KLF6 inhibited MFN1 and MFN2 protein levels as well as increased Drp1 protein levels (P<0.001) (fig. 3C-D). Moreover, mitochondrial swelling, rupture, and vacuolation occurred when KLF6 was over expressed compared with the control group (fig. 3E). Nevertheless, the knockdown of KLF6 had the reverse effect (P<0.001) (fig. 3F-G). Therefore, the results confirmed that KLF6 can promote mitochondrial fission.

(A-D) CFs were transfected with pcDNA3.1-KLF6 and the expression of KLF6, Drp1, MFN1 and MFN2 was analyzed by Western blot. (E) CFs were transfected with pcDNA3.1-KLF6 and the morphology of mitochondria was observed by electron microscope. (F-G) CFs were transfected with si-Ctl or si-KLF6 for 24 h. The expression of Drp1, MFN1 and MFN2 was analyzed by Western blot. The data are presented as the mean ± SD. Data are analyzed using one-way ANOVA followed by Tukey's post-hoc test. ***P<0.001 versus the corresponding control.

(A-B) CFs were treated with TGF-β1 (10 ng/mL) for the indicated times. The expression of Drp1, MFN1 and MFN2 was analyzed by Western blot. (C-D) CFs were treated with different doses of TGF-β1 for 24 h. The expression of Drp1, MFN1 and MFN2 was analyzed by Western blot. (E) CFs were treated with TGF-β1 and the morphology of mitochondria was observed by electron microscope. (F-G) CFs were treated with Mdivi-1 and then treated or not with TGF-β1 (10 ng/mL) for 24 h. The expression of Drp1, Collagen I, Collagen III and α-SMA was analyzed by Western blot. The expression of Drp1, MFN1, and MFN2 was analyzed by Western blot. The data are presented as the mean ± SD. Data are analyzed using one-way ANOVA followed by Tukey's post-hoc test. *P<0.05, **P<0.01, ***P<0.001, ###P<0.001 versus the corresponding control.

Mdivi-1 inhibits TGF-β1-induced myocardial fibrosis and mitochondrial division

The above results confirmed that myocardial fibrosis increased the expression of KLF6 and promoted mitochondrial fission. We would like to further explore whether myocardial fibrosis induced by TGF-β1 is related to mitochondrial fission. Then, we found that TGF-β1 increased Drp1 expression at the protein levels in a manner dependent on both the dose and time of exposure. In addition, TGF-β1 decreased MFN1 and MFN2

expressions (P<0.05, P<0.01, P<0.001) (fig. 4A-D). In addition, mitochondrial swelling, rupture and vacuolation occurred after TGF-β1 treatment (fig. 4E). Next, we observed that the Collagen I, Collagen III, α-SMA and the mitochondrial fission protein Drp1, were down regulated in the Mdivi-1 group compared to the control group. Whereas the expression of myocardial fibrosis markers and mitochondrial fission protein was markedly augment in the Mdivi-1+TGF-β1 group (P<0.001) (fig. 4F-G). The results suggested that TGF-β1-induced myocardial fibrosis is associated with mitochondrial fission.

KLF6 induces mitochondrial dysfunction and accelerates the progression of myocardial fibrosis

To illustrate that KLF6 induces mitochondrial dysfunction and promotes the advancement of myocardial fibrosis, we conducted KLF6 knockdown experiments and treated the cells with Mdivi-1. The Western blot analysis revealed that the Collagen I, Collagen III and α-SMA were markedly reduced in the Mdivi-1 group compared to the control group. (P<0.001) (fig. 5A-B). Moreover, the knockdown of KLF6 could further inhibit the expression of myocardial fibrosis markers. The results revealed that siKLF6 can suppress myocardial fibrosis by inhibiting mitochondrial fission.

(A-B) CFs were transfected with si-KLF6 and then treated or not with Mdivi-1 (10 mM) for 24 h. The expression of Collagen I, Collagen III and α-SMA was analyzed by Western blot. The data are presented as the mean ± SD. Data are analyzed using one-way ANOVA followed by Tukey's post-hoc test. ***P<0.01, ###P<0.001 versus the corresponding control.

Down-regulation of KLF6 can inhibit Keap1/Nrf2 signaling pathway activation

Oxidative stress is closely related to mitochondrial dynamics (Ma *et al*., 2021). In further experiments, we investigated the potential molecular pathways associated with KLF6 in regulating oxidative stress and Keap1/Nrf2 pathway was detected. We confirmed that the TGF-β1 group markedly increased the expression of the Keap1 protein and reduced the expression of the Nrf2 protein. In addition, KLF6 knockdown suppressed the Keap1 protein level. However, Nrf2 was obviously increased (P<0.05, P<0.001) (fig. 6A-B).

Next, we examined the impact of siKLF6 on Mitochondrial ROS generation. Immunofluorescence staining demonstrated that the level of Mitochondrial reactive oxygen species in TGF-β1-treated CFs was markedly increased. On the contrary, the KLF6 knockdown inhibited the level of Mitochondrial reactive oxygen species (fig. 6C). These results suggested that siKLF6 can inhibit Keap1/Nrf2 signaling pathway and inhibited the production of Mitochondrial reactive oxygen species. The above results suggested that knocked-down KLF6 can inhibit myocardial fibrosis by inhibiting mitochondrial division and reducing ROS production.

(A-B) CFs were transfected with si-KLF6 and then treated or not with TGF-β1 (10 ng/mL) for 24 h. The expression of Keap1 and Nrf2 was analyzed by Western blot. The data are presented as the mean ± SD. Data are analyzed using one-way ANOVA followed by Tukey's post-hoc test. *P<0.05, ***P<0.001, ###P<0.001 versus the corresponding control. (C) Immunofluorescent staining of mitochondria ROS and the cells treated or not with KLF6 and TGF-β1. Scale bars=200μm.

DISCUSSION

Mitochondria are highly dynamic organelles that respond to cellular stress by maintaining structural and functional integrity through continuous fusion and fission (Giacomello *et al.*, 2020; Tokuyama *et al*., 2023). A lot of studies have verified that oxidative stress and inflammation are the core events of MF (Yang *et al*., 2021; Zhou *et al.*, 2014; Suthahar *et al*., 2017). After myocardial infarction, aseptic inflammation and oxidative stress of myocardial tissue were induced, which activated CFs and then induced MF (Prabhu and Frangogiannis 2016; Talman and Ruskoaho, 2016; van and Turner 2013). Mitochondria serve as both the primary source of reactive oxygen species and the principal target of oxidative damage (Kokkinopoulou *et al.*, 2021; Rizwan *et al*., 2020; Kasai *et al.,* 2020). Therefore, the intervention of CFs' mitochondrial dynamic balance is an effective treatment strategy to inhibit the formation of fibroblasts in heart disease.

KLF6 is an intercellular communication signaling molecule between cardiomyocytes and cardiac fibroblasts (Balligand 2015), which plays a complex regulatory role in the process of cardiac fibrosis (Cullingford *et al*., 2008). Compared with the wild type, the degree of cardiac fibrosis in KLF6+/- mice was reduced (Sawaki *et al*., 2015). In this study, we observed a significant upregulation of KLF6 and Drp1 in ISO-induced myocardial fibrosis models, while MFN1 and MFN2 were significantly down-regulated. In addition, knocking down KLF6 inhibited TGF-β1-induced myocardial fibrosis. These results suggested that KLF6 is involved in the regulation of mitochondrial homeostasis induced by myocardial fibrosis.

Mitochondrial division inhibitor 1 (Mdivi-1), a quinazolinone derivative, has been employed as an inhibitor of mitochondrial fission protein. Mdivi-1 effectively suppressed the expression of Collagen I, Collagen III, α-SMA and Drp1 induced by TGF-β1. This study demonstrates that Mdivi-1 can inhibit myocardial fibrosis and mitochondrial fission. These findings are consistent with previous studies (Ding *et al.*, 2022). Importantly, we found that the knockdown of KLF6 enhanced the inhibitory effect of Mdivi-1 on myocardial fibrosis, suggesting that KLF6 can play an anti-myocardial fibrosis role by mediating mitochondrial division.

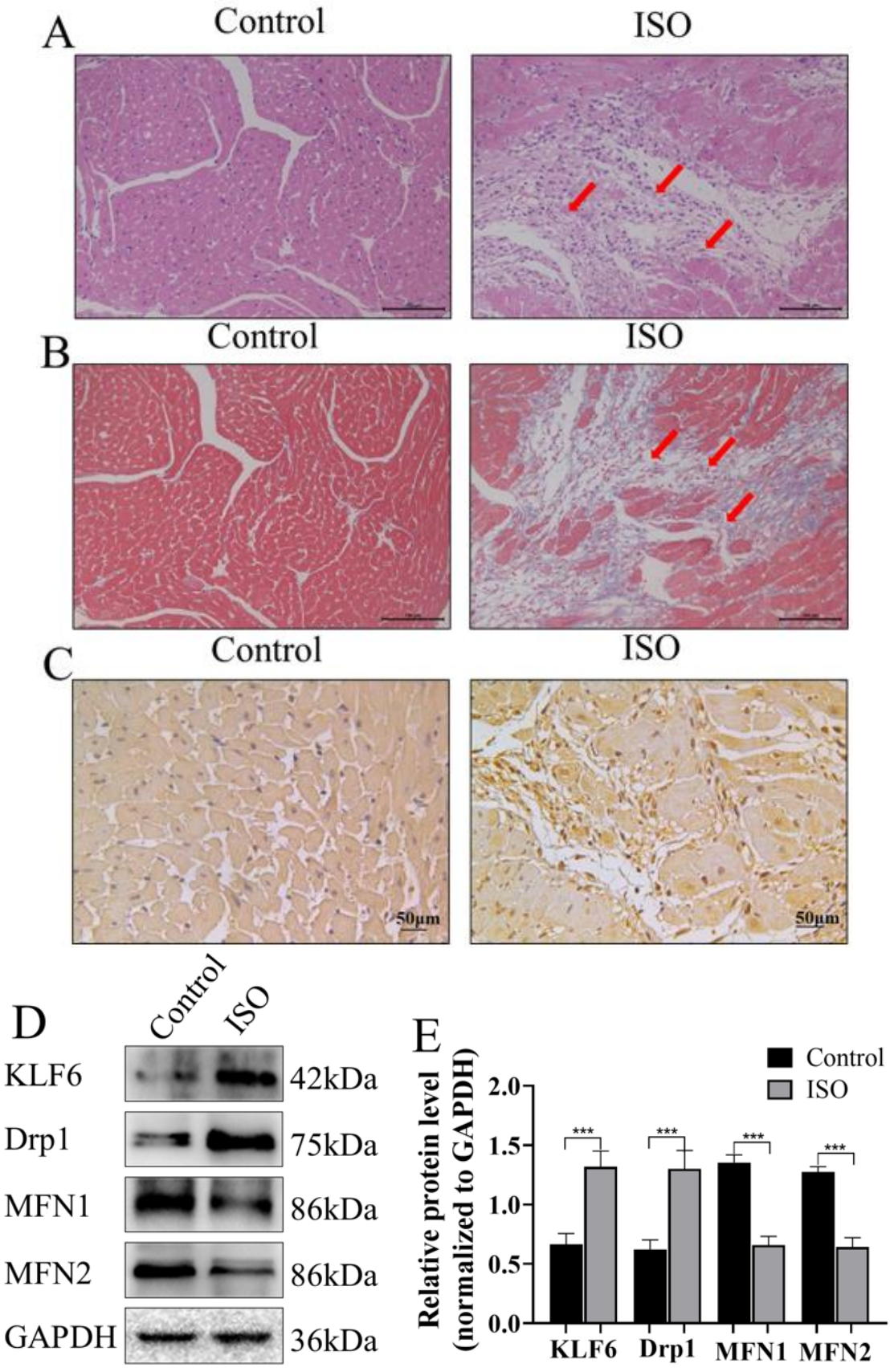

Fig. 1: ISO-induced myocardial fibrosis models induced KLF6 upregulation and mitochondrial division

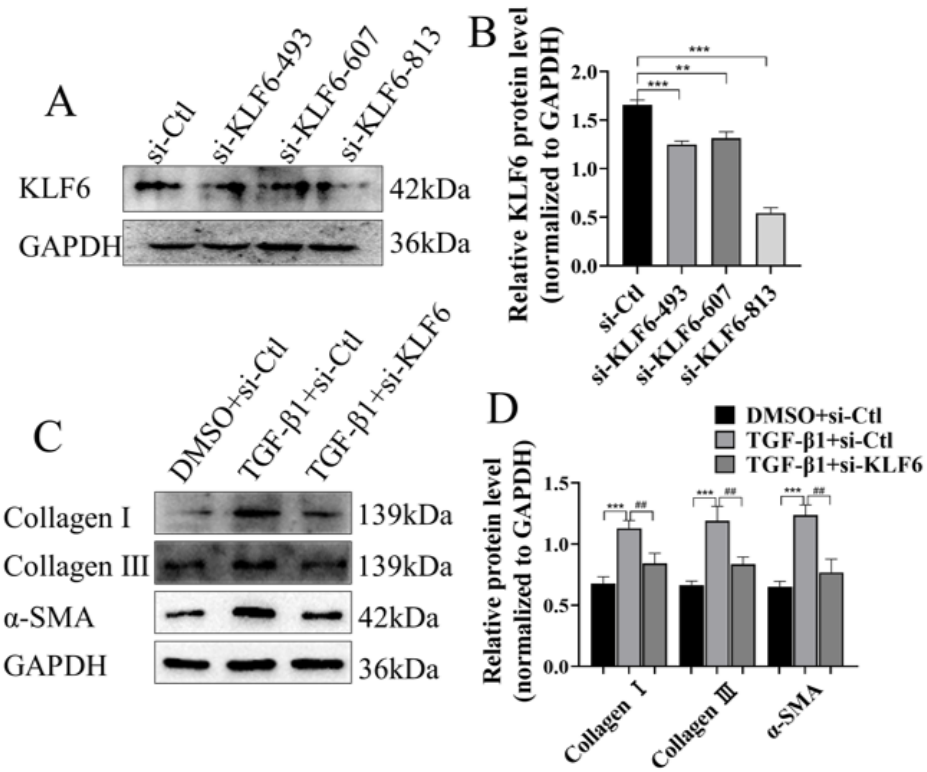

Fig. 2: siKLF6 can inhibit myocardial fibrosis

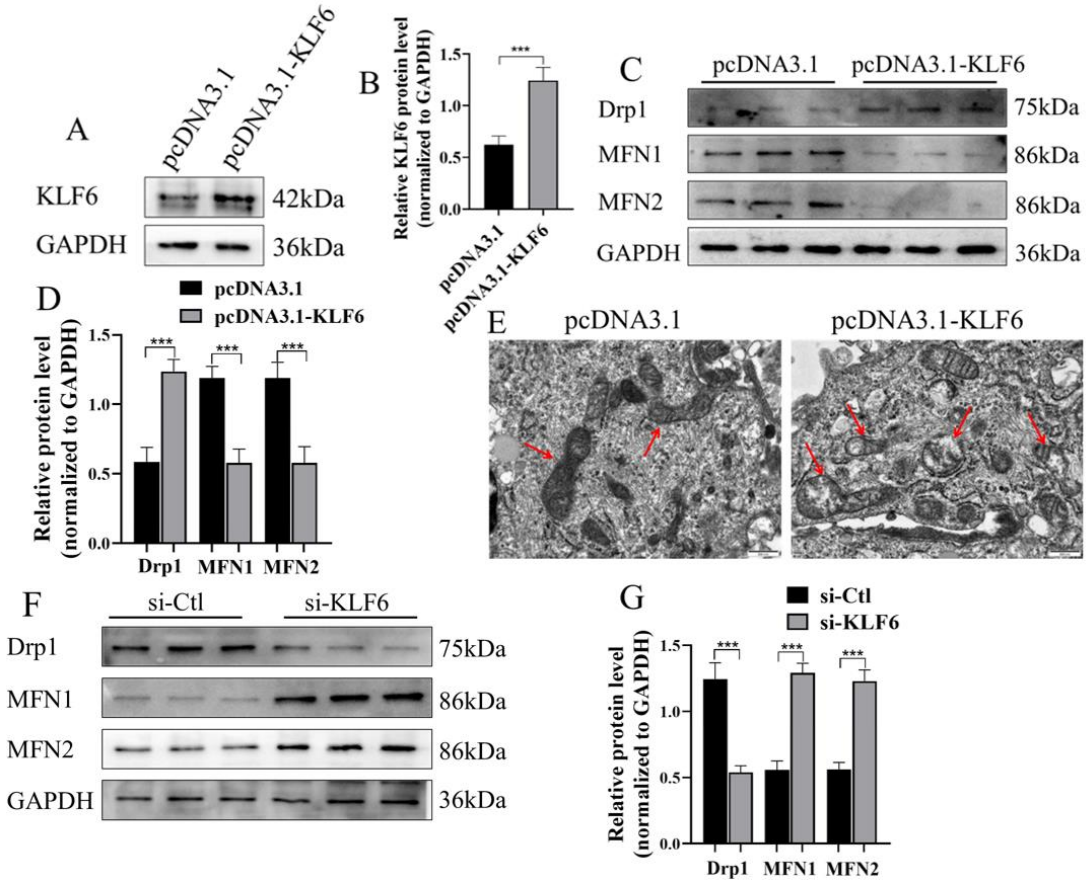

Fig. 3: siKLF6 inhibits mitochondrial division

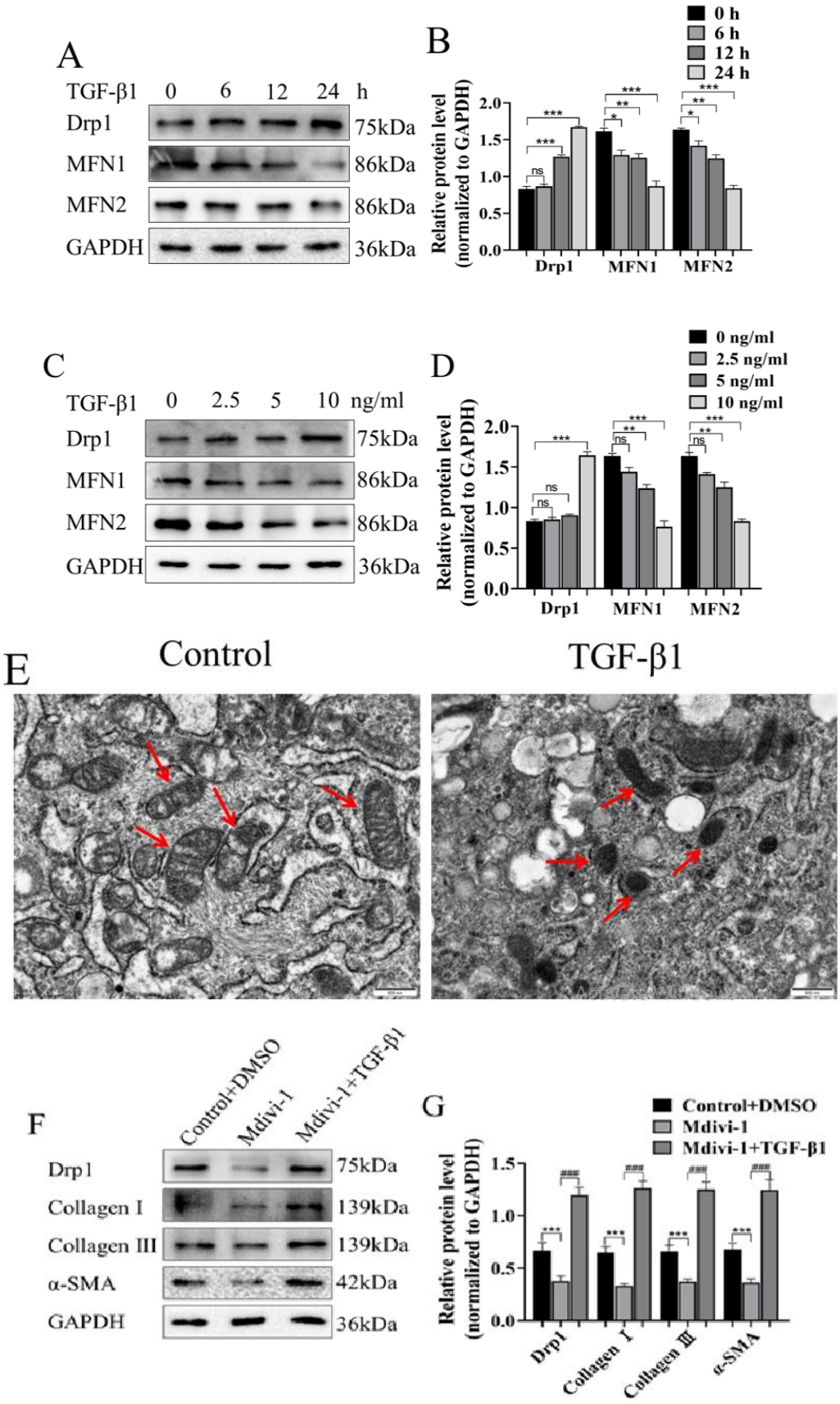

Fig. 4: Inhibition of mitochondrial division can inhibit myocardial fibrosis

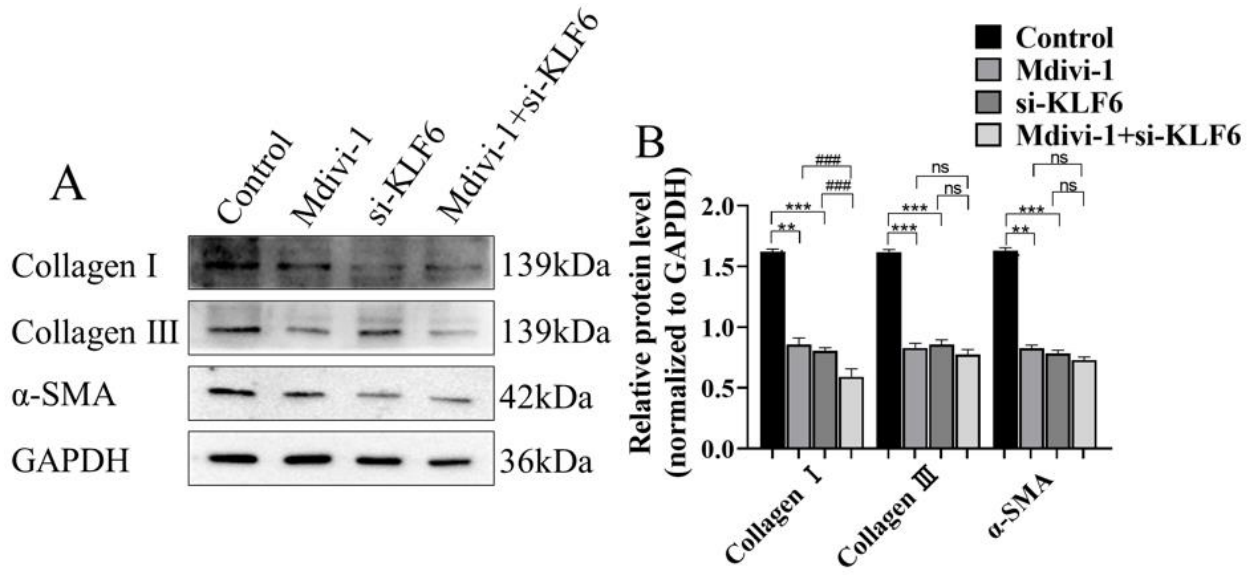

Fig. 5: siKLF6 inhibits mitochondrial division and thus myocardial fibrosis

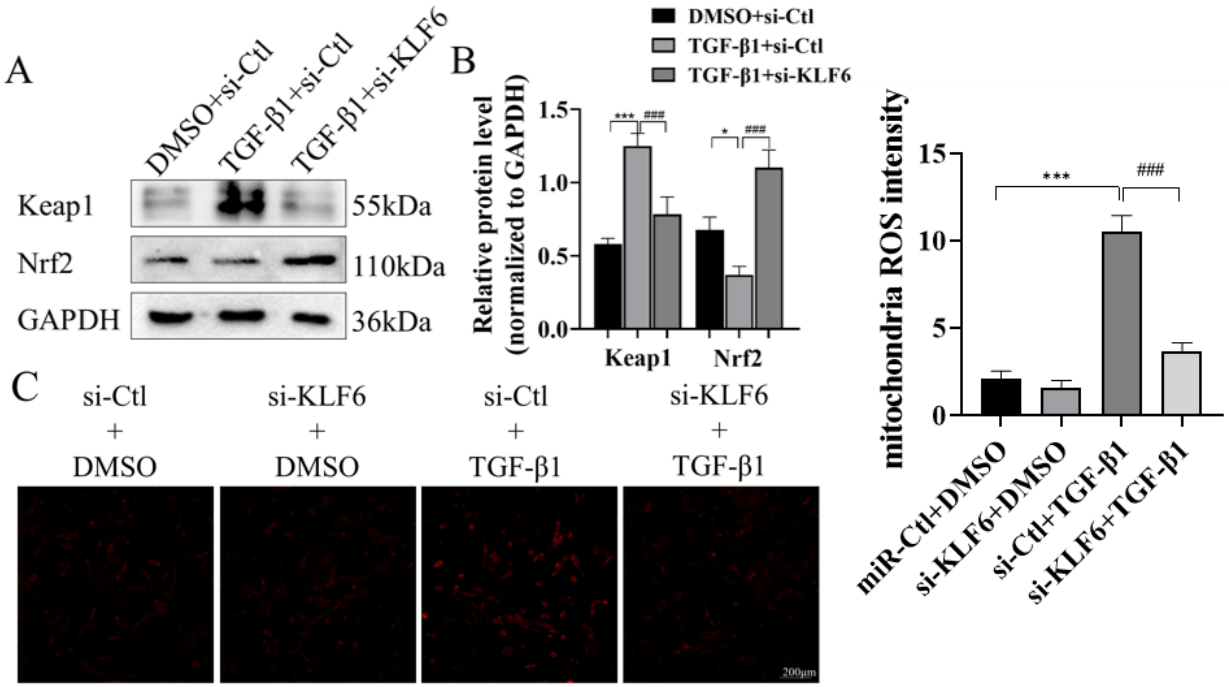

Fig. 6: siKLF6 inhibits myocardial fibrosis by inhibiting Keap1/Nrf2 pathway

Oxidative stress is defined as a state in which cell damage and excessive production of ROS in the body exceed the inherent antioxidant defense ability of cells, thereby damaging proteins and DNA (Li *et al*., 2019). Previous studies have confirmed that ROS can produce pro-fibrosis factors, such as Transforming growth factor β (TGF-β). Further promoting the phenotype transformation of fibroblasts into myofibroblasts. Here, we found that the activation of the Keap1/Nrf2 signaling pathway induced by TGF-β1 resulted in a significant upregulation of Keap1 expression and a significant downregulation of Nrf2 expression. Knocking down KLF6 inhibited the expression of Keap1 and significantly alleviated the inhibition of Nrf2. Next, we examined the effect of KLF6 on mitochondrial reactive oxygen species and observed in CFs that knocking down KLF6 could inhibit TGF-β1-induced mitochondrial reactive oxygen species expression. However, oxidative stress can destroy the Dynamic equilibrium of mitochondria and aggravate the occurrence of myocardial fibrosis. Therefore, the above results indicate that KLF6 can regulate the Dynamic equilibrium of mitochondria by targeting the Keap1/Nrf2 signal pathway to improve myocardial fibrosis.

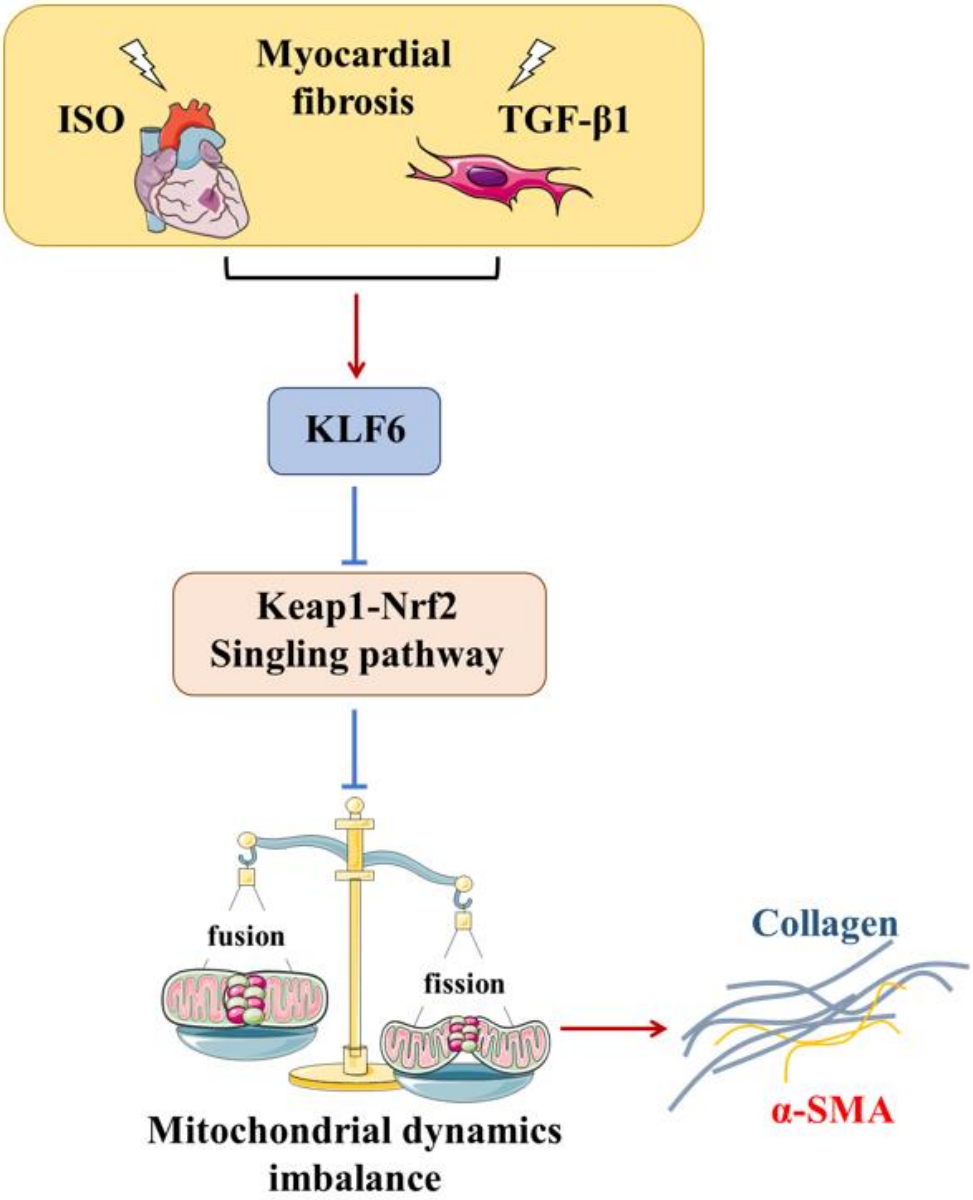

Fig. 7: Schematic representation for KLF6-mediated Keap1-Nrf2 signaling pathway inhibiting mitochondrial dynamic imbalance during myocardial fibrosis.

The major findings in the present study are that 1) myocardial fibrosis induces upregulation of KLF6 expression and promotes mitochondrial fission. 2) KLF6 promotes mitochondrial fission. 3) knocked down of KLF6 can inhibit myocardial fibrosis by inhibiting Keap1/Nrf2 pathway and mitochondrial division.

Investigating the molecular mechanism of mitochondrial fission is beneficial for understanding and potentially preventing or treating myocardial fibrosis. As a Multifunctional zinc-finger transcription factor, the function of KLF6 in the mitochondrial fission of CFs is not clear. The aim of this research was to elucidate the function and molecular mechanism of KLF6 in regulating mitochondrial fission. Mitochondrial dynamics-related marker proteins and signaling molecules were assessed via Western blot analysis following knockdown and overexpression of KLF6 in CFs. The results indicated that KLF6 could aggravate mitochondrial fission and the Keap1/Nrf2 pathway molecules. To further explore whether the influence of KLF6 on mitochondrial fission might impact cardiac fibroblasts, we constructed TGF-β1-induced CFs and ISO-induced mouse models. The results showed that KLF6 can inhibit myocardial fibrosis by inhibiting mitochondrial division and reducing ROS production. These findings underscore the regulatory mechanism of mitochondrial fission by KLF6 and suggest the potential utility of this gene as a target for preventing heart disease (fig. 7).

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