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ORIGINAL CONTRIBUTION

Dickkopf-3 protects against cardiac dysfunction and ventricular remodelling following myocardial infarction

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Abstract Dickkopf-3 (DKK3) is a secreted glycoprotein of the Dickkopf family (DKK1–4) that modulates Wnt signalling. DKK3 has been reported to regulate cell development, proliferation, apoptosis, and immune response. However, the functional role of DKK3 in cardiac remodelling after myocardial infarction (MI) has not yet been elucidated. This study aimed to explore the functional significance of DKK3 in the regulation of post-MI remodelling and its underlying mechanisms. MI was induced by surgical left anterior descending coronary artery ligation in transgenic mice expressing cardiac-specific DKK3 and DKK3 knockout (KO) mice as well as their non-transgenic and $DKK3^{+/+}$ littermates. Our results demonstrated that after MI, mice with DKK3 deficiency had increased mortality, greater infarct size, and exacerbated left ventricular (LV) dysfunction. Significantly, at 1 week post-MI, the hearts of DKK3-KO mice exhibited increased apoptosis, inflammation, and LV remodelling compared with the hearts of their $DKK3^{+/+}$ littermates. Conversely, DKK3 overexpression led to the opposite phenotype after infarction. Similar results were observed in cultured neonatal rat cardiomyocytes exposed to hypoxia in vitro. Mechanistically, DKK3 promotes cardioprotection by interrupting the ASK1–JNK/p38 signalling cascades. In conclusion, our results indicate that DKK3 protects against the development of MI-induced cardiac remodelling via negative regulation of the ASK1–JNK/p38 signalling pathway. Thus, our study suggests that DKK3 may represent a potential therapeutic target for the treatment of heart failure after MI.

Keywords DKK3 · Myocardial infarction · Cardiac remodelling · Apoptosis · Inflammation · ASK1

M.-W. Bao, Z. Cai, and X.-J. Zhang are co-first authors.

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Introduction

Myocardial infarction (MI) resulting from coronary artery occlusion is a leading cause of morbidity and mortality worldwide [42]. After acute MI, the loss of ventricular muscle triggers a host of molecular and cellular remodelling that initiate and subsequently modulate reparative responses including inflammation, hypertrophy, fibrosis, and the formation of a discrete collagen scar [11, 34]. Over the long term, post-infarct pathological events would contribute to left ventricular (LV) remodelling, chamber dilatation, cardiac dysfunction, and thus result in the

development of chronic heart failure and death [8, 40]. Despite pharmacological (e.g., β-blockers, renin–angiotensin–aldosterone system inhibitors) and technical advances for the treatment of heart diseases, the incidence of heart failure keeps increasing, and mortality remains relatively high [8, 22]. Therefore, it is of critical importance to explore the maladaptive mechanisms underlying heart failure and to develop novel therapeutic strategies that can effectively inhibit this deleterious process.

The Dickkopfs (DKKs) are a conserved family that consists of four members (DKK1–4) and a unique DKK3-related gene, DKKL1 (soggy) [33]. DKKs were originally described as negative regulators of Wnt signalling that are known to play important roles in regulating development and carcinogenesis [1, 29]. Similar to other DKK members, DKK3, also known as reduced expansion in immortalised cells (REIC), possesses an N-terminal signal peptide and contains two conserved cysteine-rich domains separated by a linker region [25]. The structural domains of DKK3 allow its binding to co-factors and its regulatory effects on development, homeostasis, and various pathological conditions. DKK3 is widely expressed in adult tissues, with the highest levels found in the heart and brain [33]. Accumulating studies have shown that DKK3 plays an essential role in the regulation of embryonic development, including development of the neural epithelium, limb bud, and bone, particularly in regions of epithelial–mesenchymal transformation [4, 31]. Additionally, DKK3 has been shown to be down-regulated in several types of human cancer, and its overexpression can inhibit cell proliferation [24, 35]. Most recently, we reported that DKK3 protects against pressure overload-induced cardiac hypertrophy and fibrosis in mice [52]. These results suggest that DKK3 plays a vital role in the pathophysiological function of heart. Unlike the pathogenesis and progression of cardiac hypertrophy, MI involves a wide range of pathological alterations, in particular cardiomyocyte apoptosis and inflammation [18, 48]. Until now, it has remained unclear whether DKK3 plays a role in the pathological processes that occur after MI. Hence, the investigation of the pathophysiological contribution of cardiac DKK3 expression to LV remodelling and cardiac dysfunction following MI would provide valuable understanding on heart failure.

In the current study, DKK3 was significantly down-regulated in both failing human hearts and infarcted mouse hearts. Applying DKK3-overexpressing and DKK3-deficient mice, we found that MI-induced cardiac remodelling was significantly exaggerated in DKK3-deficient mice but was blunted in DKK3-overexpressing mice. We further discovered that the DKK3-mediated cardioprotective effects on post-MI remodelling were at least partially dependent on the negative regulation of the ASK1–JNK/p38 signalling pathway. This study indicates that manipulating DKK3 expression may represent a promising therapeutic strategy for MI and heart failure.

Materials and methods

Reagents

Antibodies against the following proteins were purchased from Cell Signaling Technology (MA, USA): MEK1/2 (#9122), phospho-MEK1/2 (#9154), ERK1/2 (#4695), phospho-ERK1/2 (#4370), JNK1/2 (#9258), phospho-JNK1/2 (#4668), p38 (#9212), phospho-p38 (#4511), p65 (#4764), phospho-p65 (#3033), IκBα (#4814), P-IκBα (#9246), Bcl-2 (#2870), Bax (#2772), caspase 3 (#9662), cleaved caspase 3 (#9661), and GAPDH (#2118). The antibodies against DKK3 (sc14956), ASK1 (sc7931), and phospho-ASK1 (sc101634) were purchased from Santa Cruz Inc (TX, USA). The BCA protein assay kit was purchased from Pierce (Rockford, IL, USA). Foetal calf serum (FCS) was ordered from HyClone (Waltham, MA, USA). The cell culture reagents and other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Human heart samples

All procedures that involved human samples were conformed to the principles outlined in the Declaration of Helsinki and were approved by the Renmin Hospital of Wuhan University Review Board, Wuhan, China. Left ventricle samples were collected from human patients with ischemic heart disease (IHD) who were undergoing treatment following heart transplantation [51]. Control samples were obtained from the left ventricles of non-cardiac patients who had expired due to traumatic injuries and whose hearts were unsuitable for transplantation for non-cardiac reasons [6, 20, 21]. Written informed consents were obtained from the families of all prospective heart donors.

Experimental mice

The animal protocols were approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University. DKK3-knockout (DKK3-KO) mice (129S6/SvEvTac-Dkk3<tm1Tfur>, 129 background) were generously provided by Takahisa Furukawa (Osaka Bioscience Institute, Japan) and shipped by Riken (RBRC02847). DKK3-KO mice with a pure C57BL/6J background, cardiac-specific DKK3-Tg mice (C57BL/6J background), cardiac-specific dnASK1 transgenic mice (dnASK1-Tg, C57BL/6J background), cardiac-specific conditional ASK1 transgenic mice (ASK1-Cre-Tg, C57BL/6J background), dnASK1-Tg/DKK3$^{-/-}$ mice, and ASK1-Cre-Tg/DKK3-Tg

mice (DTG) were generated as previously described [52]. Male α-MHC-DKK3-Tg mice and their non-transgenic (NTG) littermates as well as DKK3-KO mice and their $DKK3^{+/+}$ littermates (aged 8–10 weeks with body weights of 24–27 g) were used in these experiments. All of the animals were housed in an environment with controlled light cycles (12 h light/12 h dark) at 21 ± 2 °C with the relative humidity of 50 %; food and water were available ad libitum.

Left coronary artery ligation surgery

Left coronary artery ligation was performed as described previously [26, 48]. Briefly, sodium pentobarbital (50 mg/kg, i.p.) was used to anesthetise mice, and the adequacy of anaesthesia was confirmed by the absence of a reflex response to foot squeeze. A left thoracotomy was performed at the third or fourth intercostal space, the pericardium was opened, and the proximal left coronary descending (LAD) artery was then encircled under the tip of the left atrial appendage and ligated using a 7–0 silk suture. In sham-operated mice, the left coronary artery was encircled without ligation. All surgeries and subsequent analyses were performed in a blinded fashion.

Echocardiography and haemodynamic evaluation

Mice were anesthetised by inhalation of 1.5–2 % isoflurane, as described previously [19]. Echocardiography was performed to evaluate the structure and function of the left ventricle using a MyLab 30CV system (Biosound Esaote, Inc.) equipped with a 15-MHz probe. To measure the LV end-systolic diameter (LVESD), LV end-diastolic dimension (LVEDD), and LV fractional shortening (FS), M-mode tracings derived from the short axis of the left ventricle at the level of the papillary muscles were recorded; parameters were obtained from at least three beats and averaged. For the haemodynamic analysis, a 1.4-French catheter-tip micromanometer catheter (SPR-839; Millar Instruments, Houston, TX, USA) was inserted into the left ventricle via the right carotid artery to obtain invasive haemodynamic measurements. An Aria pressure–volume conductance system (MPVS-300 Signal Conditioner, Millar Instruments, Houston, TX, USA) coupled with a PowerLab/4SP A/D converter was used to continuously record the heart rates, pressure, and volume signals, which were then displayed on a personal computer.

Cardiac morphology and histomorphometric analysis

After both echocardiography and haemodynamic measurements were obtained, the anesthetised mice were immediately killed. The hearts were arrested with 10 % KCl, fixed in 10 % formalin, embedded in paraffin, and transversely sliced into 5-μm sections that were stained with haematoxylin–eosin (H&E) and picrosirius red (PSR), respectively. High-magnification light micrographs were obtained by light microscopy. H&E slides were used for morphometric analysis and for determination of the infarct size at the mid-papillary muscle level using a quantitative digital analysis system (NIH Image 1.6, National Institutes of Health, USA). The infarct size was expressed as a percentage of the total LV area. Hearts with non-transmural infarcts were excluded from subsequent analyses to minimise confounding effects on biological endpoints resulting from infarct size variability. The cross-sectional area (CSA) of the myocytes was examined based on H&E-stained sections using a quantitative digital image analysis system (Image-Pro Plus 6.0). More than 100 myocytes in the left ventricles were outlined in each group. Evidence of interstitial collagen deposition was visualised using PSR staining and analysed using the software of Image-Pro Plus 6.0.

Immunofluorescence analysis

The hearts were rapidly removed and fixed with a 10 % solution of formalin in PBS. Subsequently, the LV tissues were embedded in paraffin and cut serially from the apex to the base (5 μm). Next, paraffin sections were prepared, dried, dewaxed, hydrated, and repaired under high pressure (100 mmHg). The sections were then washed in PBS, sealed with 10 % goat serum, and incubated overnight at 4 °C with the following primary antibodies: anti-p-p65 (3037, Cell Signaling Technology), LY6G (551459, BD Biosciences, NJ, USA), and MAC-1 (ab75476; Abcam, Cambridge, UK). After washing in PBS, the sections were probed with the indicated secondary antibodies for 1 h at 37 °C and subsequently washed in PBS. The following secondary antibodies were used: goat anti-rat IgG (H + L) Alexa Fluor® 568 (A11077, Invitrogen, Carlsbad, CA, USA), and donkey anti-rabbit IgG (H + L) Alexa Fluor® 568 (A10042, Invitrogen). The nuclei were stained with DAPI (S36939, Invitrogen). Images were collected with fluorescence microscope (OLYMPUS DX51, Tokyo, Japan) using DP2-BSW software (version 2.2) and analysed with Image-Pro Plus 6.0.

Detection of cell death

Apoptotic cells were measured using terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assays with the ApopTag® Plus In Situ Apoptosis Fluorescein Detection Kit (Millipore, USA, #S7111). Histological sections were prepared and stained according to the manufacturer's instructions. The TUNEL-positive cells

in the peri-infarct zone of the heart were evaluated using a fluorescence microscope (OLYMPUS DX51) and quantified under high-power magnification (400×). More than four fields in the peri-infarct areas on each slide were randomly examined.

The cultured cells were treated with the appropriate adenoviruses for 24 h and subsequently stimulated with hypoxia for 24 h. For morphological evaluation of apoptosis or necrosis in cardiomyocytes after hypoxia, the cells were stained with Hoechst 33258/propidium iodide (PI). After treatment, the cells were washed with PBS and stained with 10 μg/ml Hoechst 33258 (H3569, Invitrogen) and 10 μg/ml PI (P4864, Sigma-Aldrich) for 15 min. The cells were then observed under a fluorescence microscope. Cell viability is presented as the number of dead (PI-stained) cells/total number of cells (Hoechst 33258-stained).

Quantitative real-time PCR and Western blot analysis

Quantitative real-time PCR and Western blotting were performed as previously described [20, 28]. Briefly, total RNA was extracted from mouse hearts and neonatal rat cardiomyocytes (NRCMs) using TRIzol (15596-018, Invitrogen), and cDNA was generated with the Transcriptor First Strand cDNA Synthesis Kit (04897030001, Roche Diagnostics, Basel, Switzerland). Transcripts were then amplified using SYBR Green (Roche), and GAPDH served as the endogenous reference gene. The real-time PCR primers used in these experiments are the same as we have described previously [19].

Western blotting was conducted using extracts from cardiac tissues and NRCMs. The protein concentration was measured using a Pierce® BCA Protein Assay Kit (23225, Thermo Scientific, MIT, USA). Each lane of an SDS-PAGE gel (Invitrogen) was loaded with 50 μg of protein for electrophoresis. Then, the proteins were transferred to a PVDF membrane (IPVH00010, Millipore, MIT, USA). The blots were incubated with the indicated primary antibodies overnight at 4 °C followed by incubation with secondary antibodies for 1 h at room temperature (25 °C). Subsequently, the membranes were treated with ECL reagents (170-5061, Bio-Rad, Hercules, CA, USA) prior to visualisation using a Bio-Rad ChemiDoc^{TM} XRS^{+} according to the manufacturer's instructions. GAPDH was used as the loading control.

Cardiomyocyte culture and infection with recombinant adenoviral vectors and induction of hypoxia

Primary NRCMs were cultured as previously described [19]. Briefly, neonatal hearts of 1- to 2-day-old Sprague–Dawley rats were removed from the thoracic cavities after euthanasia. PBS containing 0.03 % trypsin and 0.04 % collagenase type II was used to digest finely minced heart tissue. NRCMs were enriched by differential pre-plating for 2 h; seeded at a density of 1×10^5 cells/well onto gelatine-coated 6-well culture plates; and cultured in DMEM/F12 containing 20 % FCS, 0.1 mM BrdU, and penicillin/streptomycin at 37 °C for 48 h. Subsequently, the culture media were changed to serum-free DMEM/F12 for 12 h before hypoxia treatment. To induce hypoxia, cultured cardiomyocytes were incubated in a Biospherix C-Chamber (model C-274, Biospherix, Redfield, NY, USA) inside a standard culture chamber. The oxygen (O_2) concentration was maintained at 5 % inside the C-Chamber, and the carbon dioxide concentration was maintained at 5 % by mixing N_2 and CO_2 with a ProOx 110 oxygen controller and a ProCO$_2$ CO_2-controller (Biospherix, NY, USA). Meanwhile, the control group without hypoxia was maintained in normal atmosphere of 5 % CO_2 and 95 % air at 37 °C. The Cell Counting Kit-8 (CCK8, CK04, Donjindo, Tokyo, Japan) assay was used to determine cell viability, and lactate dehydrogenase (LDH) release was assessed by a colorimetric LDH cytotoxicity assay (G1782, Promega, Madison, WI, USA) according to the manufacturer's protocol.

To knockdown or overexpress DKK3, AdshDKK3 or AdDKK3 adenoviruses were generated and infected into NRCMs as described previously [52]. Similarity, AddnASK1 (a dominant negative mutant of ASK1) and AdcaASK1 (constitutively activation of ASK1) were infected into AdshDKK3- and AdDKK3-infected NRCMs to down-regulate and overexpress ASK1, respectively. The AdshRNA and AdGFP, a similar recombinant adenovirus expressing GFP, were used as the non-targeting controls.

Statistical analysis

The data are presented as the mean ± standard deviation (SD). Differences among groups were assessed using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Comparisons between two groups were performed using Student's t test. A value of $P < 0.05$ was considered statistically significant.

Results

DKK3 is down-regulated in human IHD hearts and murine infarcted hearts

To investigate the potential role of DKK3 in the pathological processes that develop after MI, we first examined whether DKK3 expression levels were altered in diseased

hearts. Western blotting showed that DKK3 protein levels were dramatically down-regulated in hearts collected from patients with IHD compared with that of normal donors ($P < 0.05$, Fig. 1a). The DKK3 expression levels were further examined in the infarcted hearts of wild-type mice that had undergone coronary ligation or a sham operation. As shown in Fig. 1b, consistent with the observations in the human heart, the protein levels of DKK3 were significantly decreased during the first 1 week post-MI and continuously reduced in the following days (28 days; $P < 0.05$ vs. sham operation). Collectively, the alterations of DKK3 levels in both human IHD hearts and murine infarcted hearts indicate that DKK3 may be involved in post-infarction cardiac dysfunction and ventricular remodelling.

DKK3 ameliorates post-infarction outcomes of MI

The varied DKK3 levels in the post-infarction hearts promote us to specifically identify the effects of altered DKK3 on the cardiac dysfunction induced by infarction, and thus the MI model was respectively established on the global DKK3-KO ($DKK3^{-/-}$) or cardiac-specific DKK3-Tg mice, as well as their littermate controls. It is important to note that, under basal conditions, neither the depletion nor overexpression of DKK3 impaired the normal pathological conditions of cardiac structure or function [52]. In the sham groups, all the mice parallelly survived surgery and were alive at the end of the observation period. However, cardiac rupture caused high rate of sudden death, and the survival rate up to 1 week after MI reached to 48.4 % in the $DKK3^{+/+}$ group, which was even lower in $DKK3^{-/-}$ mice (25.9 %; Fig. 2a). In addition, H&E staining indicated that the ratio of infarct size at 1 week after coronary ligation was significantly larger in $DKK3^{-/-}$ mice than their $DKK3^{+/+}$ controls, as shown in Fig. 2b, c (47.8 % in $DKK3^{-/-}$/MI group vs. 36.7 % in $DKK3^{+/+}$/MI group, $P < 0.05$). Furthermore, using serial echocardiography and haemodynamic measurements, cardiac function was evaluated in mice from indicated groups at 1 week post-MI. We found that the MI-induced cardiac dysfunction was strikingly aggravated by DKK3 deficiency, as indicated by lower fractional shortening percentage (FS%), ejection fraction percentage (EF%), dP/dt_{max}, dP/dt_{min}, and end-systolic pressure (ESP) in $DKK3^{-/-}$ mice than those in $DKK3^{+/+}$ mice, while the end-diastolic pressure (EDP) of $DKK3^{-/-}$ was higher than that in $DKK3^{+/+}$ mice but no statistic difference (Fig. 2d). However, the heart rate has no significant difference between $DKK3^{+/+}$ and $DKK3^{-/-}$ (Fig. 2d).

To further confirm the effects of DKK3 on the outcomes of post-infarction, we generated DKK3-Tg mice lines with full-length mouse DKK3 cDNA under the control of the α-myosin heavy chain promoter, and the mouse line possessing the highest levels of DKK3 expression in the heart (Tg4) was selected for the following experiments [52]. In contrast to DKK3 deficiency, overexpression of DKK3 obviously elevated the survival rate from 51.6 to 61.8 % in the first 1 week after MI (Fig. 2e), while the infarct size induced by MI was significantly reduced from 35.3 to 23.4 % by DKK3 overexpression (Fig. 2f, g). In addition, MI-induced cardiac dysfunction was significantly improved in DKK3-Tg mice 1 week after MI, as evidenced by improvements of FS%, EF%, dP/dt_{max}, dP/dt_{min}, ESP, and EDP (Fig. 2h). Collectively, these data indicate that the DKK3 dramatically suppressed MI-induced mortality, blunted infarct size enlargement, and improved cardiac function.

DKK3 attenuates MI-induced cell death

Given that cardiomyocytes damage could be induced at the initiation of MI due to the hypoxia and glycogen

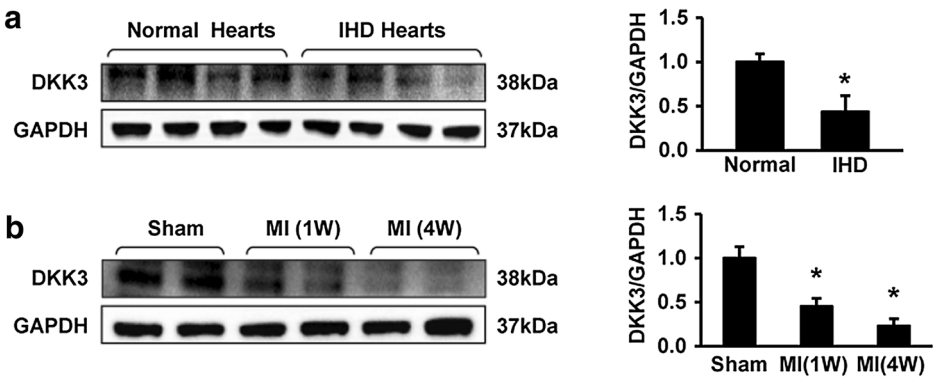
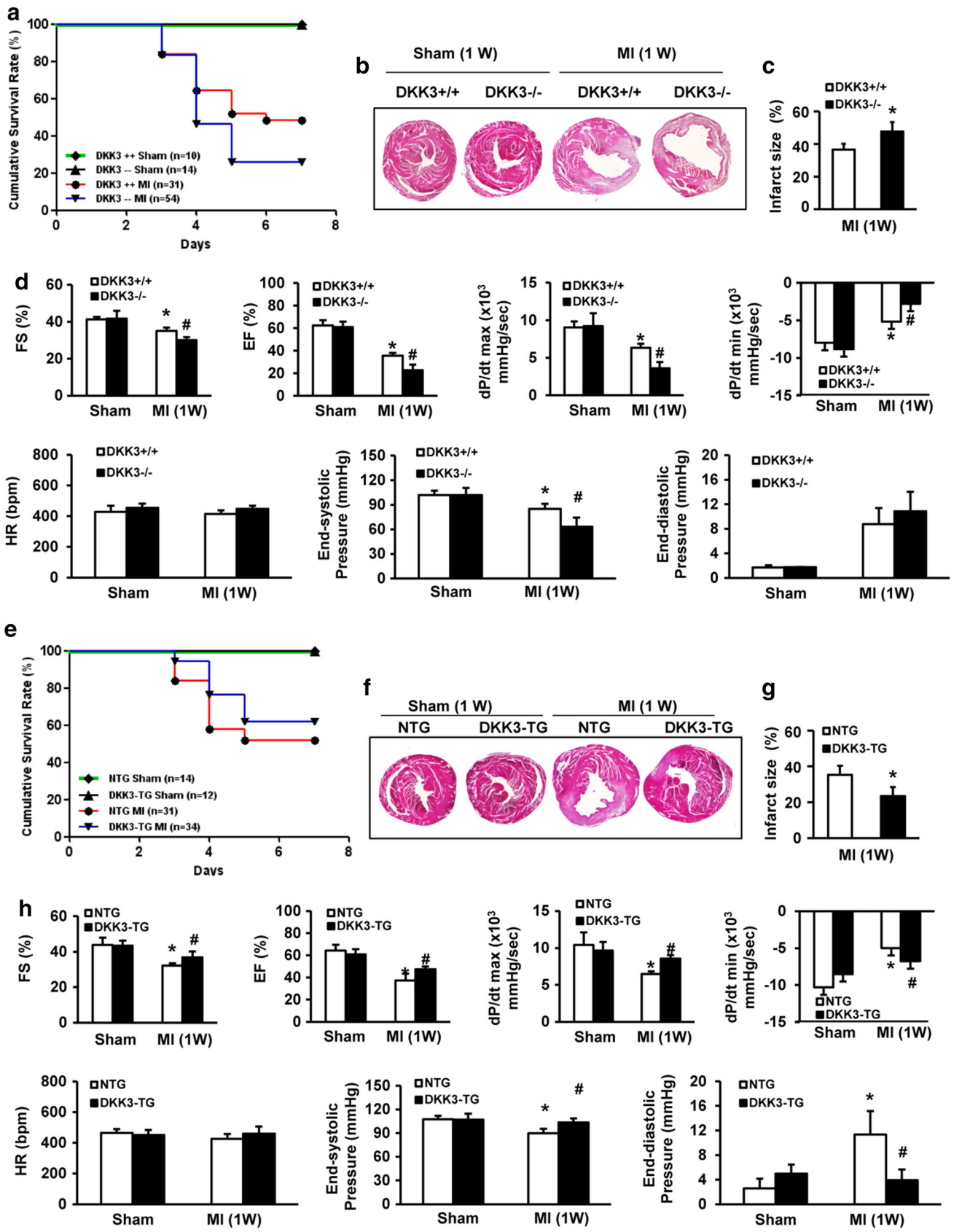


Fig. 1 Decreased DKK3 expression in human IHD hearts and murine post-infarction hearts. **a** Representative Western blots of DKK3 in the hearts of normal donors and patients with ischemic heart disease (*IHD*; $n = 4$; $^*P < 0.05$ vs. donor hearts). **b** Western blot analysis of DKK3 in wild-type mouse hearts after MI at the indicated times ($n = 4$; $^*P < 0.05$ vs. sham). *Left* representative blots. *Right* bar graphs illustrating quantitative results


a
Cumulative Survival Rate (%)
Days
DKK3 ++ Sham (n=10)
DKK3 -- Sham (n=14)
DKK3 ++ MI (n=31)
DKK3 -- MI (n=54)
b
Sham (1 W)
MI (1 W)
DKK3+/+
DKK3-/-
c
Infarct size (%)
MI (1W)
d
FS (%)
EF (%)
dP/dt max (x10³ mmHg/sec)
dP/dt min (x10³ mmHg/sec)
HR (bpm)
End-systolic Pressure (mmHg)
End-diastolic Pressure (mmHg)
Sham
MI (1W)
e
NTG Sham (n=14)
DKK3-TG Sham (n=12)
NTG MI (n=31)
DKK3-TG MI (n=34)
f
NTG
DKK3-TG
g
h

◀**Fig. 2** DKK3 attenuates mortality, infarct size expansion, and dysfunction after MI. **a**, **e** Kaplan–Meier survival analysis of **a** DKK3$^{+/+}$ and DKK3$^{-/-}$ mice or **e** NTG and DKK3-Tg mice in the first 1 week after MI. Heart sections from infarcted **b** DKK3$^{+/+}$ and DKK3$^{-/-}$ mice or **f** NTG and DKK3-Tg mice at 1 week after MI were stained with haematoxylin and eosin (H&E; $n = 6$–8). Quantification of relative infarct size in heart sections of **c** DKK3$^{+/+}$ and DKK3$^{-/-}$ mice or **g** NTG and DKK3-Tg mice at 1 week after MI ($^{*}P < 0.05$ vs. DKK3$^{+/+}$/MI or NTG/MI). Echocardiographic and haemodynamic results for **d** DKK3$^{+/+}$ and DKK3$^{-/-}$ mice or **h** NTG and DKK3-Tg mice at 1 week post-MI ($n = 6$–8, $^{*}P < 0.05$ vs. DKK3$^{+/+}$/sham or NTG/sham; $^{\#}P < 0.05$ vs. DKK3$^{+/+}$/MI or NTG/MI)

consumption, the extents of apoptosis were detected through TUNEL assays on the peri-infarct tissues. At 1 week post-MI, we found that a larger number of TUNEL-positive nuclei existed in the DKK3$^{-/-}$ hearts compared with that in DKK3$^{+/+}$ hearts (Fig. 3a). In contrast, the TUNEL-positive nuclei were much less in DKK3-Tg hearts than that of NTG hearts (Fig. 3b). Considering that the pro-apoptotic factors (Bax and cleaved caspase 3) and anti-apoptotic genes (Bcl-2) are largely involved and mediate the progression of apoptosis, the expression levels of these apoptosis-related proteins were examined. Western blot analyses revealed that the expressions of the Bax and cleaved caspase 3 were significantly increased while the expression of the Bcl-2 was decreased in DKK3-null hearts compared with DKK3$^{+/+}$ mice (Fig. 3c). Conversely, DKK3 overexpression markedly suppressed Bax and cleaved caspase 3 expressions and preserved Bcl-2 expression (Fig. 3d).

To determine the potentially direct influence of altered DKK3 expression on cardiomyocyte damage during MI,

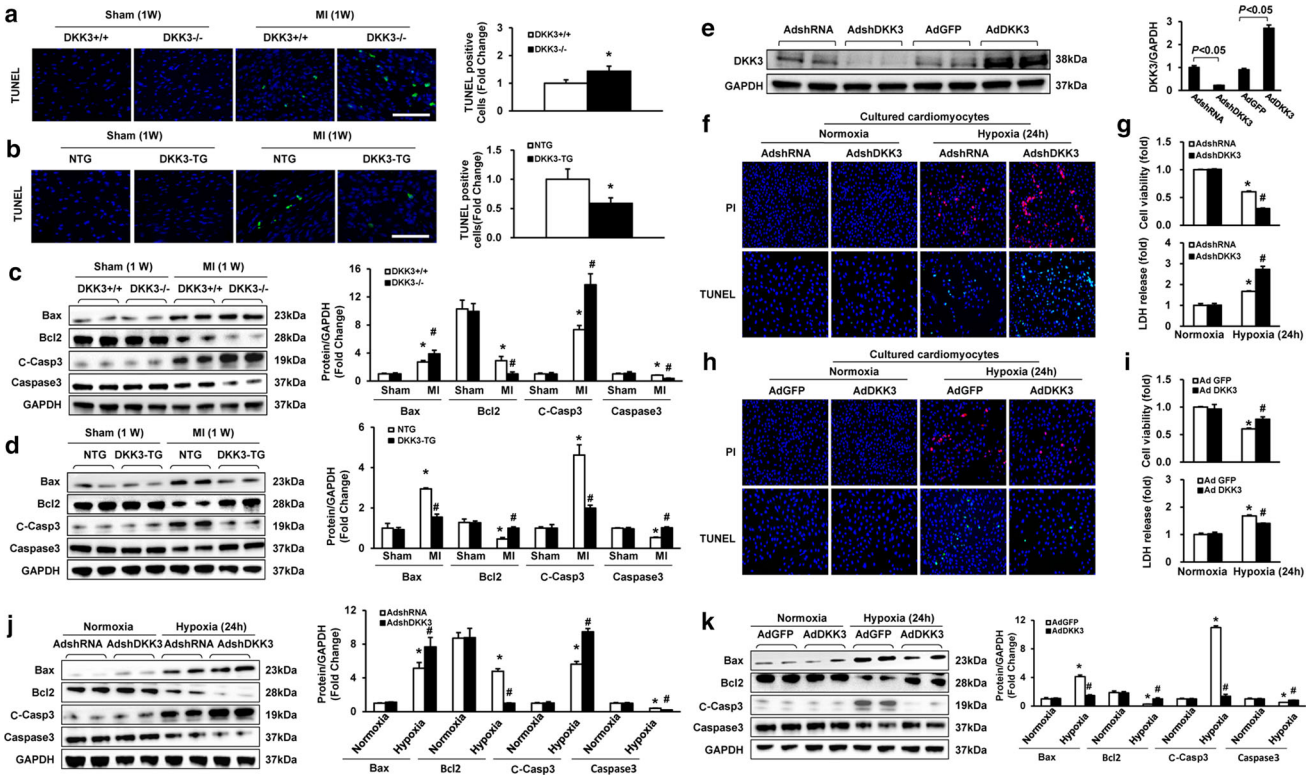


Fig. 3 DKK3 protects against apoptosis and regulates apoptosis-related genes in response to MI. TUNEL staining and quantitation in the hearts of **a** DKK3$^{+/+}$ and DKK3$^{-/-}$ or **b** NTG and DKK3-Tg mice at 1 week post-MI ($n = 4$, $^{*}P < 0.05$ vs. DKK3$^{+/+}$/MI or NTG/MI). Representative Western blots and quantitation of Bax, Bcl-2, and cleaved caspase 3 in the heart tissue of **c** DKK3$^{+/+}$ and DKK3$^{-/-}$ mice or **d** NTG and DKK3-Tg mice at 1 week after sham or MI surgery ($n = 4$, $^{*}P < 0.05$ vs. DKK3$^{+/+}$/sham or NTG/sham; $^{\#}P < 0.05$ vs. DKK3$^{+/+}$/MI or NTG/MI). **e** Representative Western blot of DKK3 expression levels in NRCMs infected with AdshRNA, AdshDKK3, AdGFP or AdDKK3, respectively. **f**, **h** Representative merged images of NRCMs infected with **f** AdshDKK3 or **h** AdDKK3 and treated with hypoxia for 24 h (magnification, ×200). *Blue* nuclei, *Red* PI positive staining, *Green* TUNEL-positive staining. **g**, **i** Cell counting kit-8 (CCK8; cell viability) and Lactate dehydrogenase (LDH; cell toxicity) assays were performed in **g** DKK3-knockdown or **i** DKK3-overexpressing cells after exposure to hypoxia for 24 h. The *blots* represent three independent experiments. $^{*}P < 0.05$ vs. AdshRNA/normaxia or AdGFP/normaxia; $^{\#}P < 0.05$ vs. AdshRNA/hypoxia or AdGFP/hypoxia. **j**, **k** Representative Western blots and quantitation of Bax, Bcl-2, and cleaved caspase 3 expression in cells transfected with **j** AdshDKK3 or **k** AdDKK3 after exposure to hypoxia for 24 h. The *blots* represent three independent experiments. $^{*}P < 0.05$ vs. AdshRNA/normoxia or AdGFP/normoxia; $^{\#}P < 0.05$ vs. AdshRNA/hypoxia or AdGFP/hypoxia. *Left* representative blots. *Right* quantitative results

cultured NRCMs were infected with either AdshDKK3 to knockdown DKK3 or AdDKK3 to overexpress DKK3 and exposed to hypoxia for 24 h (Fig. 3e). Consistent with the in vivo results, our in vitro data showed that down-regulation of DKK3 promoted hypoxia-induced cell damage, as evidenced by the increased number of PI- and TUNEL-positive cells, decreased cell viability, and increased lactate dehydrogenase (LDH) release (Fig. 3f, g), whereas overexpression of DKK3 blocked the cytotoxicity of cardiomyocytes induced by hypoxia (Fig. 3h, i). Accordingly, the expressions of pro-apoptotic markers (Bax and cleaved caspase 3) were markedly increased in AdshDKK3-infected cardiomyocytes (Fig. 3j) and dramatically reduced in AdDKK3-infected myocytes (Fig. 3k) compared with their controls, respectively. Meanwhile, under hypoxic conditions, the expression of the anti-apoptotic protein Bcl-2 was almost completely blocked after infection with AdshDKK3 and significantly enhanced in the AdDKK3-infected cells (Fig. 3j, k). Thus, our in vivo and in vitro results consistently suggest that DKK3 could regulate apoptosis-related gene expression involved in the progress of MI, and thus ameliorates MI-induced cell death.

DKK3 inhibits MI-induced inflammatory responses

Given that inflammation is a major determinant of the myocardial healing process [10], we next examined whether DKK3 could affect the inflammatory response in the heart after MI. The inflammatory cell infiltrations into the infarcted border zone were characterised using immunofluorescence analyses. Compared with the sham-operated mice, obvious infiltrations of MAC-1-positive macrophages and LY6G-positive neutrophiles were observed in the hearts of $DKK3^{+/+}$ and NTG mice at 1 week after MI, which were even higher in the hearts of $DKK3^{-/-}$ mice and dramatically decreased by DKK3 upregulation (Fig. 4a, b).

Importantly, based on ample evidences that activation of canonical NF-κB, especially the p65 subunit, profoundly regulates inflammatory response in cardiac injury [12, 13], we investigated the activation of the NF-κB pathway. The in situ immunofluorescence staining exhibited that the P-p65-positive cells were significantly increased in $DKK3^{-/-}$ ischemic hearts, but dramatically diminished in DKK3-Tg hearts compared with their controls (Fig. 4a, b). For the expression levels of proteins involved in NF-κB signalling, as described in Fig. 4c, the phosphorylated protein levels of IκBα and p65 were increased at 1 week post-MI in $DKK3^{+/+}$ mice, and these increases were even greater in $DKK3^{-/-}$ mice. However, the activation of this signalling was dramatically blunted in DKK3-Tg mice compared with NTG controls post-MI injury (Fig. 4d). The above results reveal that DKK3-mediated anti-inflammatory effect on MI might be attributable to the inactivation of the NF-κB pathway.

DKK3 ameliorates adverse LV hypertrophy and fibrosis in mice after MI

Increasing evidences have suggested that heart failure is a frequent complication of MI that is associated with pathological cardiac hypertrophy and fibrosis [46]. Therefore, we determined whether DKK3 would interfere with MI-triggered cardiac remodelling. As expected, at 1 week after MI, $DKK3^{-/-}$ mice exhibited obvious increases of the ratios of heart weight (HW)/body weight (BW), lung weight (LW)/BW, and HW/tibia length (TL) compared with $DKK3^{+/+}$ mice (Fig. 5a). H&E staining revealed that cardiomyocytes in $DKK3^{-/-}$ mice had an increased CSA compared with cardiomyocytes in $DKK3^{+/+}$ mice at 1 week after MI (Fig. 5b, c). Meanwhile, using PSR staining and analysis, a dramatically enhanced interstitial fibrosis was observed in $DKK3^{-/-}$ hearts subjected to MI compared with the control group (Fig. 5b, d). Coincided with the aggravated cardiac hypertrophy and fibrosis, the increases of LVEDd and LVESd levels in $DKK3^{-/-}$ mice were enhanced compared with those of $DKK3^{+/+}$ mice (Fig. 5e). Additionally, the mRNA levels of hypertrophic markers [atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and β-myosin heavy chain (β-MHC)] and fibrotic markers [collagen I, collagen III and connective tissue growth factor (CTGF)] were much higher in $DKK3^{-/-}$ mice compared with $DKK3^{+/+}$ mice at 1 week after MI (Fig. 5f, g), indicating a remarkable deterioration in cardiac remodelling by DKK3 depletion. Conversely, both the MI-induced hypertrophic and fibrotic responses in NTG mice were significantly attenuated by DKK3 overexpression, which were demonstrated by the blocked heart enlargement, reduced LV collagen volume and the inhibited activation of corresponding remodelling markers (Fig. 6a–g). Taken together, these data indicate that the presence of DKK3 in the heart alleviates adverse LV remodelling induced by MI.

DKK3 inhibits MI-induced activation of the JNK1/2 and p38 pathways

The above results demonstrated that DKK3 could protect against post-infarction functionally and architecturally cardiac remodelling. However, the underlying mechanism by which DKK3 exerts its anti-cardiac remodelling effect remains unknown. Growing evidences demonstrate that the MAPK signalling cascade plays an important role in heart disease, especially in apoptosis, inflammation, and hypertrophy [30]. Therefore, immunoblotting was performed to

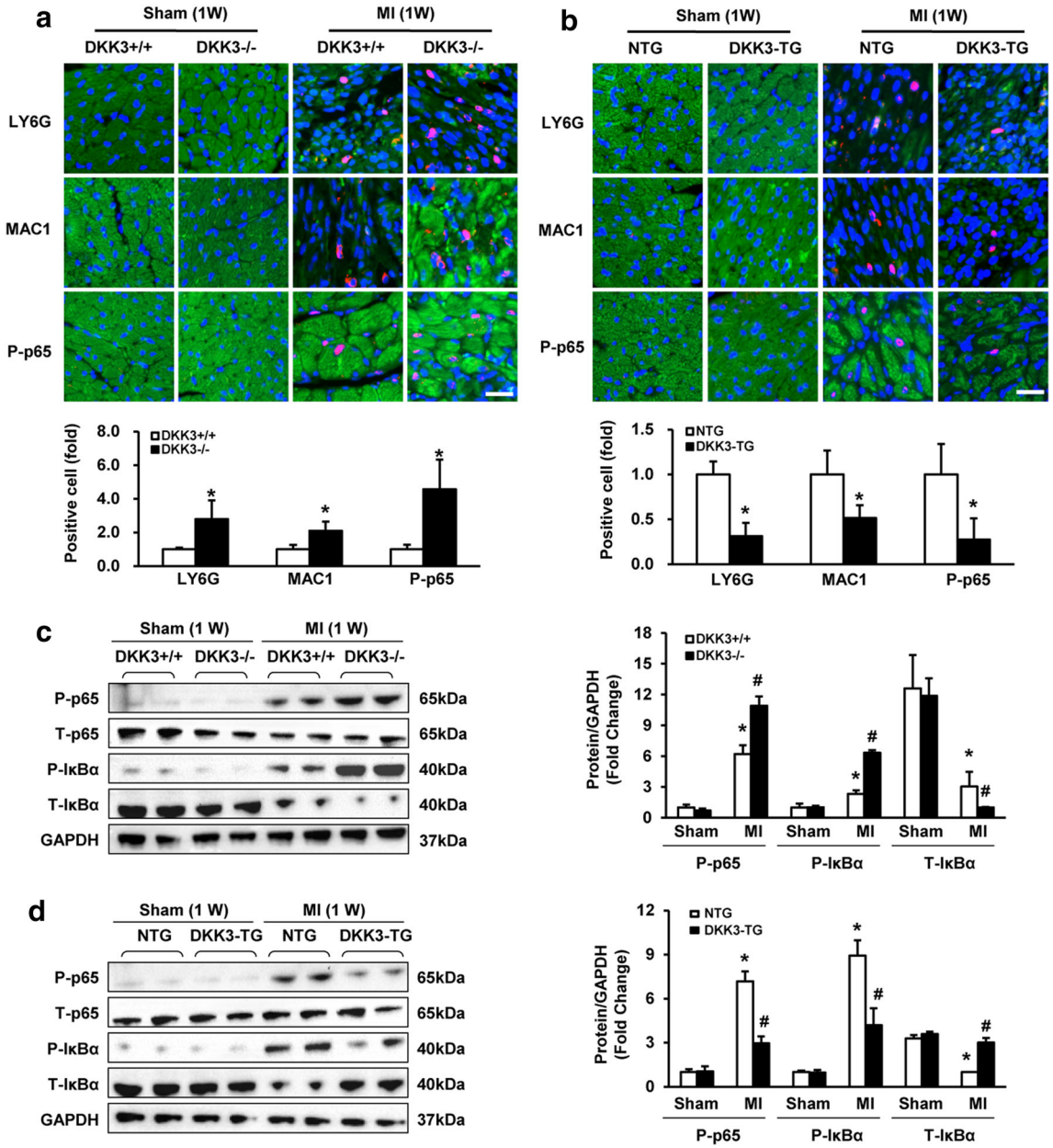


Fig. 4 DKK3 inhibits inflammatory responses following MI. **a**, **b** Immunofluorescence (IF) staining and quantitative analysis showing the number of P-p65-, LY6G-, and MAC-1-positive cells in the heart cross-sections of **a** DKK3$^{+/+}$ and DKK3$^{-/-}$ mice or **b** at NTG and DKK3-Tg mice 1 week after MI ($n = 4$, $^{*}P < 0.05$ vs. DKK3$^{+/+}$/MI or NTG/MI. *Top* representative image. *Bottom* quantitative results. **c**, **d** Representative Western blots and quantitation of p65 and IκBα in heart tissue of **c** DKK3$^{+/+}$ and DKK3$^{-/-}$ mice or **d** NTG and DKK3-Tg mice at 1 week after MI ($n = 4$, $^{*}P < 0.05$ vs. DKK3$^{+/+}$/sham or NTG/sham; $^{\#}P < 0.05$ vs. DKK3$^{+/+}$/MI or NTG/MI). *Left* representative blots. *Right* quantitative results

investigate the possible involvement of MAPK pathways in DKK3-associated molecular events during MI progression. Although the levels of phosphorylated MEK1/2, ERK1/2, JNK1/2, and p38 were significantly increased in hearts after MI, only the activations of JNK1/2 and p38 were more pronounced in DKK3$^{-/-}$ mice than in DKK3$^{+/+}$ mice (Fig. 7a). Based on the results obtained from DKK3$^{-/-}$ hearts, we next investigated whether DKK3 overexpression negatively affected the levels of phosphorylated JNK1/2 and p38 in response to MI. As shown in Fig. 7b, the MI-induced activation of JNK1/2 and p38 was almost completely blocked in DKK3-Tg hearts. To exclude potential in vivo compensatory mechanisms, we utilised cultured NRCMs to examine the effect of DKK3 on JNK1/2 and p38 signalling in response to hypoxic stimulation. Western blotting showed that the hypoxia-induced

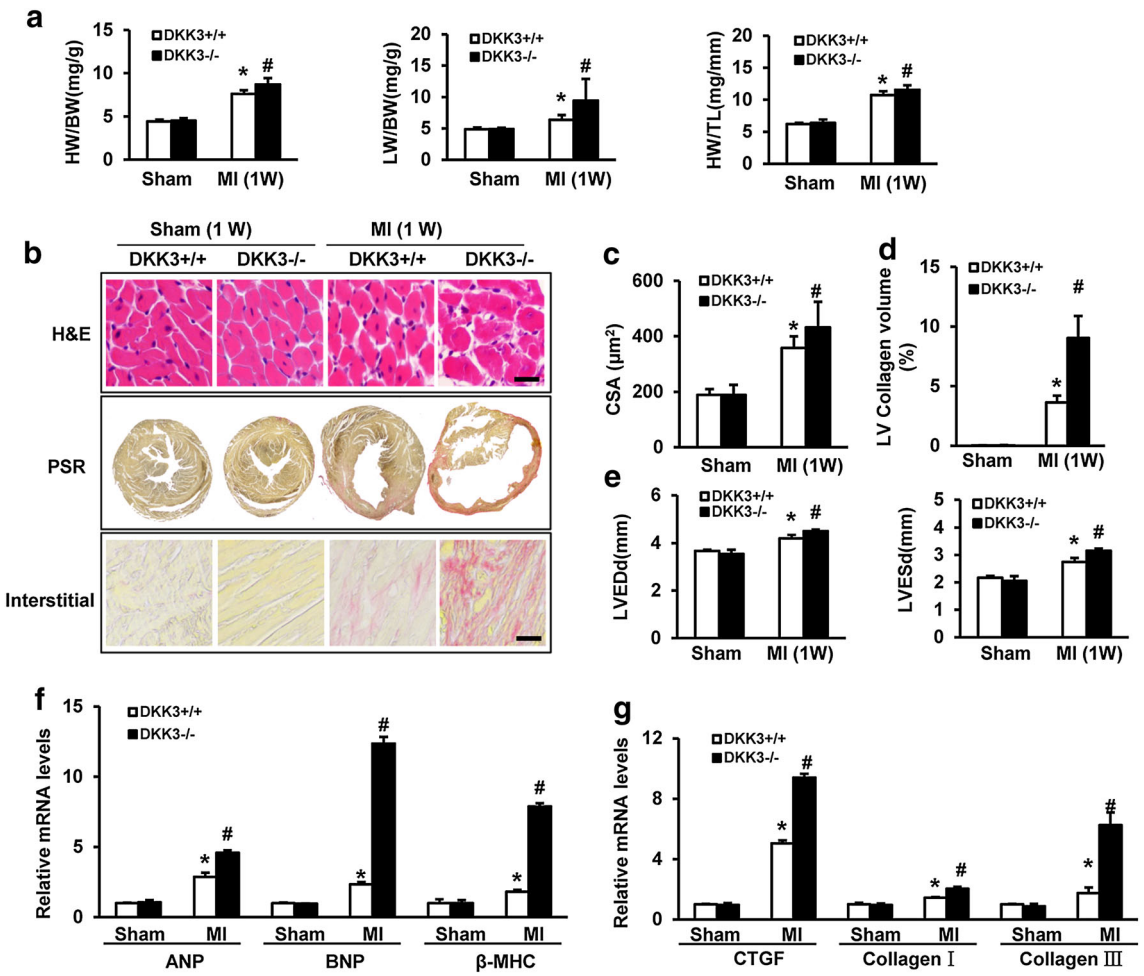


Fig. 5 DKK3 deficiency exacerbates MI-induced cardiac remodelling in mice. **a** Statistical analysis of the following ratios in the mice from DKK3$^{+/+}$ and DKK3$^{-/-}$ groups (n = 12–13): heart weight (*HW*)/body weight (*BW*), lung weight (*LW*)/BW, and HW/tibia length (*TL*). **b** Representative images of heart sections from DKK3$^{+/+}$ and DKK3$^{-/-}$ mice at 1 week after sham or MI surgery. The sections were stained with H&E or PSR (n = 5–8; *scale bars* 20 μm for H&E staining, 50 μm for PSR staining). **c** Statistical analysis of the cross-sectional area (*CSA*, n = 100 + cells per experimental group). **d** Statistical analysis of the LV collagen volume (%) (n = 25 + fields per experimental group). **e** The LVEDD and LVESD levels of mice from DKK3$^{+/+}$ and DKK3$^{-/-}$ groups (n = 12–13). **f** The relative mRNA levels of ANP, BNP, and β-MHC in left ventricular samples from DKK3$^{+/+}$ and DKK3$^{-/-}$ mice (n = 4). **g** The relative mRNA levels of CTGF, collagen I, and collagen III in samples from DKK3$^{+/+}$ and DKK3$^{-/-}$ mice (n = 4). $^{*}P < 0.05$ vs. DKK3$^{+/+}$/sham; $^{\#}P < 0.05$ vs. DKK3$^{+/+}$/MI

activation of JNK1/2 and p38 was enhanced in DKK3-knockdown cells (Fig. 7c), whereas this activation was inhibited by DKK3 overexpressing (Fig. 7d). Our results demonstrate that the DKK3-induced anti-remodelling function upon MI is largely associated with the inhibitory effects on the activation of JNK1/2 and p38 signallings in hearts.

DKK3 suppresses MI-induced cardiac remodelling in an ASK1-dependent manner

Previous studies have demonstrated that ASK1 phosphorylation is largely responsible for the activation of JNK1/2 and p38 cascades, and thus regulates stress-induced remodelling in the heart [27, 49]. To further investigate whether DKK3 affects JNK1/2 and p38 activation directly or indirectly by inhibiting upstream components of the signalling pathway, we assessed the regulatory role of DKK3 in ASK1 activation. Our results showed that DKK3$^{-/-}$ mice exhibited higher levels of ASK1 phosphorylation compared with DKK3$^{+/+}$ mice (Fig. 8a). In contrast, the MI-induced increase in the level of ASK1 phosphorylation was significantly attenuated in DKK3-Tg mice compared with NTG controls (Fig. 8b). Consistently, in vitro data demonstrated that hypoxia-induced ASK1 phosphorylation was markedly increased in AdshDKK3-infected cardiomyocytes (Fig. 8c), but dramatically decreased by AdDKK3 infection (Fig. 8d). To further verify

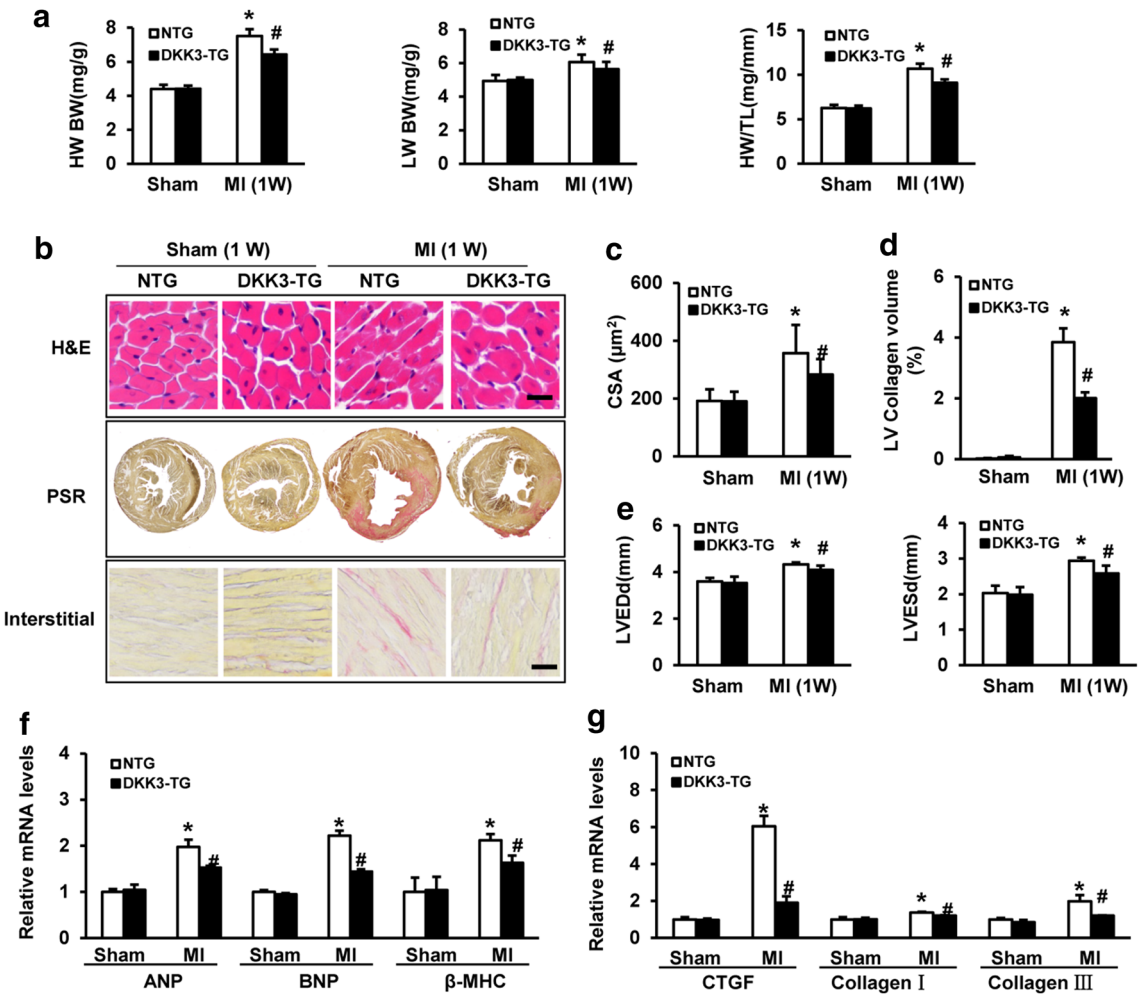


Fig. 6 DKK3 overexpression ameliorates adverse LV remodelling in mice after MI. **a** The ratios of HW/BW, LW/BW, and HW/TL in the NTG and DKK3-Tg mice ($n = 12$–13). **b** Representative H&E and PSR images of heart sections from NTG and DKK3-Tg mice at 1 week post-sham or MI operation. **c** Statistical analysis of the cross-sectional area (*CSA*, $n = 100 +$ cells per experimental group) of NTG and DKK3-Tg mice at 1 week post-MI. **d** Statistical analysis of the LV collagen volume (%) ($n = 25 +$ fields per experimental group) of NTG and DKK3-Tg mice at 1 week post-MI. **e** The LVEDD and LVESD levels of mice from NTG and DKK3-Tg groups. **f**, **g** The relative mRNA expressions of **f** hypertrophic markers (*ANP*, *BNP*, and *β-MHC*) and **g** fibrotic markers in left ventricular samples from NTG and DKK3-Tg mice ($n = 4$). $^{*}P < 0.05$ vs. NTG/sham; $^{\#}P < 0.05$ vs. NTG/MI

the influence of ASK1–JNK/p38 signalling cascades on DKK3-induced cardioprotection, AddnASK1 and AdcaASK1 were infected into AdshDKK3- and AdDKK3-infected NRCMs, respectively. As shown in Fig. 8e, f, AddnASK1 significantly reduced JNK/p38 signalling cascades, while AdcaASK1 enhanced the phosphorylated JNK and p38 expressions. Importantly, upon hypoxia stimulation, co-infection of AddnASK1 with AdshDKK3 almost completely abolished the reduced cell viability and elevated LDH release by DKK3 knockdown (Fig. 8g). In contrast, the ameliorated cytotoxicity in AdDKK3-infected cardiomyocytes induced by hypoxia was largely reversed by overexpression of caASK1 (Fig. 8h). Notably, these in vitro findings were verified in an in vivo myocardial infarction (MI) mouse model. ASK1-inducible transgenes were introduced into mice with DKK3-Tg background to generate DKK3-Tg/ASK1-Cre-Tg (DTG) animal. As showed in Supplemental Fig. 1a–1f, compared with MEM-Cre controls, the infarct size, cross-sectional area, LV collagen volume, LVEDd, and LVESd were significantly increased, while FS%, EF%, dP/dt_{max}, and dP/dt_{min} were remarkably decreased in the ASK1-Cre-Tg mice after 1 week of MI. Importantly, overexpression of ASK1 in the DKK3-Tg background almost nullified the protective effects of DKK3 on cardiac remodelling induced by MI, as evidenced by larger infarct size, decreased cardiac function, and increased cardiomyocytes size and myocardial fibrosis in DTG mice (Supplemental Fig. 1a–1f). Furthermore, we were interested in whether the inactivation of ASK1 could reverse the detrimental role of DKK3

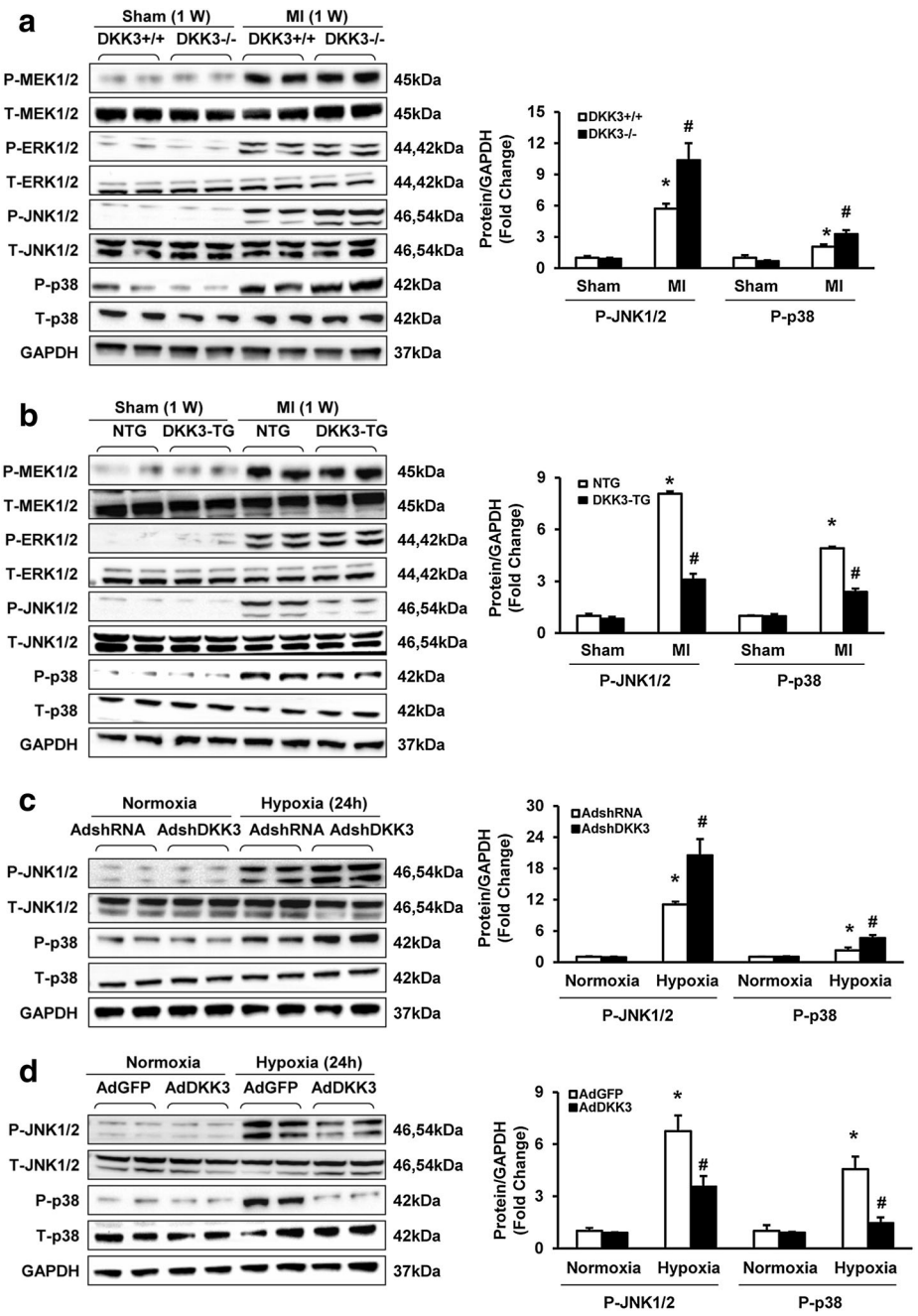


Fig. 7 DKK3 modulates MI-induced activation of the JNK1/2 and p38 pathway. **a**, **b** Representative Western blots and quantitation of total and phosphorylated MEK1/2, ERK1/2, JNK1/2, and p38 in the **a** $DKK3^{+/+}$ and $DKK3^{-/-}$ mice or in the **b** NTG and DKK3-Tg mice at 1 week after sham or MI surgery ($n = 5$, $^{*}P < 0.05$ vs. $DKK3^{+/+}$/sham or NTG/sham; $^{\#}P < 0.05$ vs. $DKK3^{+/+}$/MI or NTG/MI). **c**, **d** Representative Western blots and quantitation of phosphorylated and total JNK1/2 and p38 in **c** AdshDKK3 or **d** AdDKK3 adenoviral vector-infected cardiomyocytes after exposure to hypoxia for 24 h. The *blots* represent three independent experiments. $^{*}P < 0.05$ vs. AdshRNA/normoxia or AdGFP/normoxia; $^{\#}P < 0.05$ vs. AdshRNA/hypoxia or AdGFP/hypoxia

deficiency on cardiac dysfunction and ventricular remodelling following MI. The dnASK1, a catalytically inactive mutant form of ASK1 in which Lys-709 has been replaced by Arg, was overexpressed in the heart of DKK3 knockout mice via crossing the $DKK3^{-/-}$ mice with cardiac-specific dnASK1-Tg mice. After 1 week of MI, dnASK1 overexpression protected the mice against from injury in response to MI. Importantly, dnASK1 overexpression also dampened the adverse effects of DKK3 deficiency on cardiac remodelling and cardiac dysfunction post-MI (Supplemental Fig. 2a–2f*)*. Collectively, these data suggest that the protective role of DKK3 in cardiac remodelling after MI is at least partially dependent on the inactivation of ASK1.

Discussion

In the present study, we for the first time identified DKK3 as a negative regulator of deleterious outcomes of MI. Using gain- and loss-of-function procedures, we demonstrated that in the absence of DKK3, mortality and infarct size were significantly increased in the heart after MI,

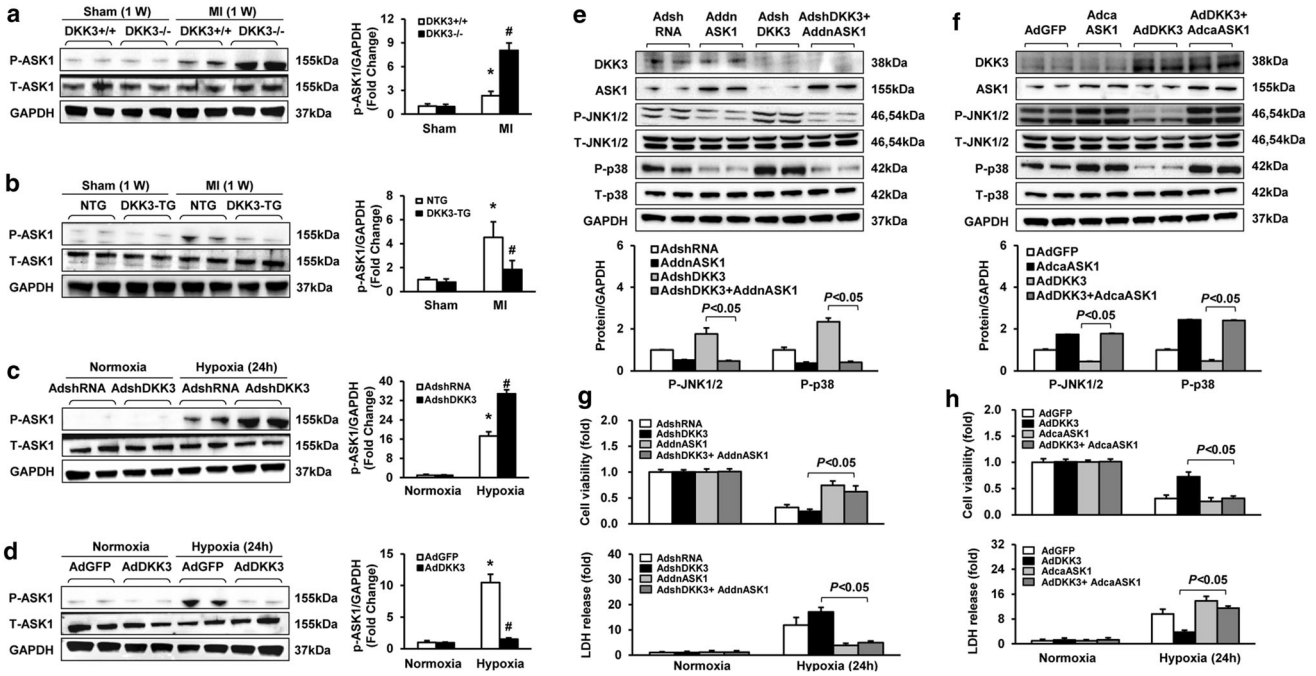


Fig. 8 DKK3 protects against deleterious outcomes of MI in an ASK1-dependent manner. **a**, **b** Representative Western blots and quantitation of phosphorylated and total ASK1 in the **a** DKK3$^{+/+}$ and DKK3$^{-/-}$ mice and in the **b** NTG and DKK3-Tg mice at 1 week after MI ($n = 6$, $^{*}P < 0.05$ vs. DKK3$^{+/++}$/sham or NTG/sham; $^{\#}P < 0.05$ vs. DKK3$^{+/+}$/MI or NTG/MI). **c**, **d** Representative Western blots and quantitation of total and phosphorylated ASK1 in **c** AdshDKK3 or **d** AdDKK3 adenoviral vector-infected cardiomyocytes after exposure to hypoxia for 24 h. The *blots* represent three independent experiments. $^{*}P < 0.05$ vs. AdshRNA/normoxia or AdGFP/normoxia; $^{\#}P < 0.05$ vs. AdshRNA/hypoxia or AdGFP/hypoxia. **e**, **f** Representative Western blots and quantitation of DKK3, ASK1, total and phosphorylated JNK1/2, and P38 after AddnASK1 and AdcaASK1 infected into AdshDKK3- and AdDKK3-infected NRCMs, respectively. **g** The cell viability and LDH release of cardiomyocytes infected with AdshRNA, AdshDKK3, and AdshDKK3 + AddnASK1 after exposure to hypoxia for 24 h. **h** The cell viability and LDH release of cardiomyocytes infected with AdGFP, AdDKK3, and AdDKK3 + AdcaASK1, and treated with hypoxia for 24 h. The *blots* represent three independent experiments

along with increased cardiomyocyte apoptosis, inflammation, LV remodelling, and functional deterioration at 1 week after MI. Conversely, DKK3 overexpression resulted in dramatic attenuations of post-infarction cardiac remodelling. More importantly, our data strongly suggest that the suppression of ASK1 and its downstream protein JNK/p38 largely account for the cardioprotective action of DKK3. Therefore, DKK3 might represent a promising therapeutic target for MI and heart failure.

DKK3 is a critical member of DKK family that ubiquitously expressed in human tissues, especially in heart and brain [33]. Given the regulatory effects of DKK3 during embryonic development and adult homeostasis [5, 52], it is not surprising that DKK3 is greatly involved in the process of cell death. However, the mediatory function of DKK3 in cell apoptosis is dual. DKK3 was initially proposed to have pro-apoptotic properties. Abarzua et al. [1] reported that DKK3 overexpression selectively induced cell death in human prostate cancer cells. Interestingly, in growth-arrested fibroblasts, the silence of DKK3 leads to apoptosis [44]. Moreover, Nakamura et al. [31] demonstrated that DKK3 prevents apoptosis by reducing caspase activity in HEK293 cells. In the present study, we observed that MI-induced apoptosis in vivo and hypoxia-induced cardiomyocytes damage in vitro were significantly exaggerated by the down-regulation of DKK3 expression compared to those of controls, whereas DKK3 overexpression obviously attenuated cardiomyocyte apoptosis following MI or hypoxia. The discrepant effects on the regulation of cell survival or death may be attributable to differences in experimental settings, cell types, stimuli, and more importantly, target genes. Our in vivo and in vitro data indicate that DKK3 significantly increased the expression of anti-apoptotic Bcl-2, and inhibited expression of cleaved caspase 3, which contribute to the anti-apoptotic effects of DKK3 in the cardiomyocytes during MI insult.

It has been well documented that innate immune and inflammatory responses are involved in the pathological processes of MI, ultimately leading to healing and scar formation. The inflammatory response in the infarcted myocardium is related to the recruitment of inflammatory cells and coordinated activation of a series of inflammatory

cascades [32, 43]. A reduction in the number of inflammatory infiltrates is associated with ameliorated myocardial injury after ischemia [39, 47]. In our present study, loss of DKK3 facilitated inflammatory cell infiltration in association with increased p-p65, MAC-1, and LY6G expression in the heart, whereas DKK3 overexpression significantly attenuated the inflammatory response at 1 week after MI. Our findings are in agreement with Papatriantafyllou et al. [35] who reported that DKK3 functions as an immune regulator and efficiently inhibits T-cell reactivation and cytokines production in response to in vitro antigen stimulation. Furthermore, NF-κB, a sensitive transcription factor that has been demonstrated to play pivotal roles in MI, is a critical element in the regulation of pro-inflammatory genes [12]. Hamid et al. [14] have demonstrated that persistent myocyte NF-κB p65 activation in heart failure exacerbates adverse remodelling through pro-inflammatory effects. Similarly, our present data showed that the phosphorylation of NF-κB p65 was markedly activated post-MI stimulation, which was significantly inhibited by DKK3 overexpression through regulating IκBα activation and degradation. Thus, we could reason that the beneficial effects of DKK3 are partially mediated through an anti-inflammatory effect on MI via inactivation of NF-κB.

In response to MI injury, the remodelling of LV, including cardiac hypertrophy and interstitial fibrosis, could increase diastolic stiffness and impair relaxation, ultimately promoting LV dysfunction and heart failure [11, 15, 34]. Our previous study indicated that DKK3 was capable of attenuating pressure overload-induced cardiac hypertrophy and fibrosis [52]. Additionally, Urashima et al. [45] found that the expression of DKK3 was dramatically changed in right ventricular remodelling induced by pulmonary stenosis. In this study, we found that DKK3-KO mice displayed significant increases in LV hypertrophy and dilatation as well as exaggerated interstitial fibrosis at 1 week after MI. In contrast, DKK3-Tg mice displayed a protective phenotype of cardiac remodelling and promoted cardiac function in response to MI. Considering that the defective containment of the inflammatory infiltrate into the non-infarcted myocardium may worsen fibrosis [32] and that apoptosis of cardiomyocytes is accompanied by the induction of interstitial fibrosis [7, 46], the blunted inflammatory and apoptotic responses might be responsible for the preventive effect of DKK3 on maladaptive LV remodelling and subsequent cardiac dysfunction.

Among the numerous molecular mechanisms involved in post-infarction architecturally and functionally cardiac remodelling, MAPK signalling pathway has been highlighted for its contribution to cardiac remodelling and heart failure [30, 38]. During cardiac remodelling process, cellular sensors could transmit extracellular stress into cytoplasm and activate the MAPK cascade that comprises a sequence of successively acting kinases, including ERK, p38, and JNK. In many cell types, the ERK cascade appears to specifically mediate cell growth and survival signals [36]. Inhibition of the ERK pathway enhances ischemia/reperfusion-induced apoptosis, and sustained activation of the ERK pathway during simulated ischemia mediates adaptive cytoprotection in cultured neonatal cardiomyocytes [50]. The p38 and JNK kinases constitute two important branches of the MAPK signalling cascade that function as specialised transducers of stress or injury responses, including responses to inflammatory cytokines, UV irradiation, heat shock, and ischemia/reperfusion. Hence, p38 and JNK kinases are sub-classified as stress-activated protein kinases (SAPKs) [2]. In contrast to ERK signalling, accumulating studies have demonstrated that the p38 and JNK families appear to be greatly involved in ischemic diseases mainly through their regulation on apoptosis [9]. Muslin et al. [30] reported that the activation of p38 and JNK1/2 cascades promoted pathological cardiac remodelling after MI that included cardiomyocyte apoptosis, inflammation, and fibrosis. Inhibition of JNK1 but not JNK2 suppresses apoptosis induced by ischemia/reoxygenation in rat cardiac myocytes [17]. Notably, Kaiser et al. [23] reported that sustained inhibition or activation of JNK both led to cell survival in separate mouse cardiac ischemia/reperfusion models, suggesting the complexity system of JNK-regulated cardiac diseases. In terms of the role of p38 in cardiac dysfunction, Barancik et al. [3] found that inhibition of the cardiac p38 using an SB203580 protected against cell death in the myocardium. However, interestingly, SB203580 treatment largely abolished the ischemic preconditioning-derived reduction of infarct size in a pig ischemia/reperfusion model [37]. Additionally, another inhibition of p38 MAP kinase, SB281832, could blunt rabbit heart failure through counteracting oxidative response and contractile dysfunction, without affecting apoptosis [16]. These studies indicated the multifaceted influences of JNK and p38 subunits in heart damage. In the present study, we found that all the three subunits of MAPK family could be activated by MI; however, the activation of both p38 and JNK1/2, but not ERK, was almost completely blocked by the cardiac-specific overexpression of DKK3 but markedly enhanced by DKK3 deficiency. These data, taken together with our previous results [52], allow us to speculate that the suppression of JNK1/2 and p38 signalling pathways may be regarded as an important mechanism through which DKK3 protects against MI-induced cardiac remodelling.

Considerable evidences indicate that ASK1 is unique amongst the MAPK kinases in that it appears to regulate cellular remodelling and apoptotic cell death through activation of MKK3/6 and MKK4/7, which activate JNK1/2

and p38, respectively [41]. Our results presented in this study revealed that MI- or hypoxia-elicited ASK1–JNK/p38 signalling activity was significantly enhanced by DKK3 deficiency, but almost completely suppressed by DKK3 overexpression. Interestingly, upon hypoxia stimulation, the reduced cell viability and enhanced LDH release of cardiomyocytes by DKK3 down-regulation were largely reversed by AddnASK1 infection, whereas the suppressed cytotoxicity by DKK3 upregulation was significantly abolished by caASK1 overexpression. Thus, we could reason that ASK1 is a critically important downstream factor of DKK3-regulated MI injury, and that the anti-remodelling effects of DKK3 on MI could be dependent on the inhibition of ASK1–p38/JNK cascade. Notably, in this study, we observed that ASK1 itself is also a crucial regulator of hypoxia-induced cytotoxicity, evidenced by the fact that AddnASK1 or AdcaASK1 alone could significantly suppress or exacerbate hypoxia-induced cell damage. Consistently, our previous studies and others demonstrated that ASK1-null mice exhibited reduced ventricular remodelling in response to angiotensin II infusion, MI, and pressure overload stimulation [49, 52], while ASK1 overexpression promoted cell death and cardiomyopathy following pressure overload stimulation and myocardial infarction [27], indicating that ASK1 is an important modulator of heart damage. However, it is hard to define which one, DKK3 or ASK1, is the more important role player, because both the alteration of their expression alone could induce significant influence on cardiac function under pathological conditions. From our results of this study, we just could conclude that ASK1 is the major downstream to mediate the role of DKK3 on myocardial infarction.

In conclusion, this study presents the first evidence that DKK3 protects against cardiac remodelling in response to MI. The underlying mechanism for the protective role of DKK3 in the development of post-infarction LV remodelling involves the inhibition of the ASK1–JNK/p38 signalling pathway. Therefore, we propose that targeting DKK3 is a promising approach for preventing or treating post-infarction cardiac remodelling and heart failure.

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Conflict of interest No conflicts of interest were declared.

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