Inhibition of microRNA-124-3p protects against acute myocardial infarction by suppressing the apoptosis of cardiomyocytes

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Abstract. The aims of the present study were to investigate the roles and underlying mechanisms of microRNA-124-3p (miR-124-3p) in the progression of acute myocardial infarction (AMI). The expression of miR-124-3p was determined via reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TargetScan analysis and a luciferase reporter assay were conducted to reveal the association between miR-124-3p and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) repressing factor (NKRF). To investigate the role of miR-124-3p in AMI, a cell model of myocardial hypoxic/ischemic injury was established by subjecting H9c2 cardiac cells to hypoxia for 48 h. The viability of cells was determined using an MTT assay, and cell apoptosis was analyzed by flow cytometry. Additionally, the expression levels of inflammatory factors [tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6] were measured via ELISA. Furthermore, gene and protein expression levels were determined by performing RT-qPCR and western blot analyses, respectively. It was revealed that the expression of miR-124-3p was significantly increased in the blood of patients with AMI and hypoxia-treated H9c2 cells. Additionally, it was demonstrated that NKRF was a direct target of miR-124-3p. The hypoxia-induced decrease in the viability of H9c2 cells and increase in cell apoptosis were eliminated by the downregulation of miR-124-3p. Furthermore, hypoxia significantly increased the levels of TNF-α, IL-1β and IL-6, whereas miR-124-3p downregulation eliminated these effects. Downregulated expression of B-cell lymphoma 2, pro-caspase 3 and pro-caspase 9 protein, and upregulated expression of cleaved caspases 3 and 9 was observed in hypoxic H9c2 cells; the altered expression of these proteins was suppressed by miR-124-3p inhibitor. Additionally, miR-124-3p inhibitor suppressed the hypoxia-induced activation of the NF-κB signaling pathway in H9c2 cells. Furthermore, it was demonstrated that the various effects of miR-124-3p inhibitor on H9c2 cells were eliminated by the small interfering RNA-mediated downregulation of NKRF. In conclusion, the results of the present study indicated that miR-124-3p downregulation protected against AMI via inhibition of inflammatory responses and the apoptosis of cardiomyocytes by regulating the NKRF/NF-κB pathway.

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Introduction

Acute myocardial infarction (AMI) is a type of severe ischemic heart disease, presenting as myocardial necrosis induced by acute and continuous ischemia and hypoxia following the acute occlusion of the coronary artery (1,2). Ventricular remodeling and heart failure are two principal factors that influence the cardiovascular event rate, long term survival and quality of life following AMI (3). AMI is characterized by acute onset, high mortality and disability, and is a major human health problem (4). Therefore, the identification of novel and effective AMI treatment strategies is required.

At present, numerous studies have reported that inflammation and the apoptosis of cardiomyocytes are involved in mediating impaired myocardial function and heart failure, thus serving important roles in the pathogenesis of AMI (5,6). Increasing evidence has indicated that a large quantity of myocardial cell apoptosis is observed in the infarct area, with partial irreversible apoptosis directly contributing to AMI-induced cardiac injury (7). Following MI, the developing apoptosis of cardiomyocytes, induced by ischemic stress in the border zone, aggravates the ventricular remodeling of the remaining active myocardium, leading to further heart failure (8,9). Therefore, the prevention of cardiomyocyte apoptosis is considered to be one strategy for preventing cardiac remodeling and heart failure progression following AMI, thereby improving the prognosis of patients. Further investigation into the mechanisms underlying AMI-induced apoptosis and antiapoptotic pathways in cardiomyocytes may aid the identification of novel targets for intervention in the clinical setting.

Previous studies have aimed to identify highly sensitive and specific markers for the timely diagnosis, prevention and control of the occurrence and development of AMI (10,11).

MicroRNAs (miRNAs/miRs) are a class of short non-coding RNAs (19-25 nucleotides in length) (12). miRNAs regulate gene expression by binding to the 3'-untranslated region (3'-UTR) of target mRNAs (12,13). A large number of miRNAs have been identified in various species (14). miRNAs serve important roles in the regulation of various cellular functions, including the differentiation, proliferation, metastasis and apoptosis of cells (15,16). Numerous studies have demonstrated that the abnormal expression of miRNAs is associated with pathological processes in various cardiovascular diseases, including AMI (17-21). A recent study reported that the expression of miR-124 was abnormally upregulated in the blood of patients with AMI (22), suggesting that it may be involved in the occurrence and development of AMI; however, the expression and roles of miR-124-3p in AMI remain to be investigated. Therefore, the aims of the present study were to investigate the role of miR-124-3p in the development of AMI and to identify the underlying molecular mechanisms.

Materials and methods

Clinical samples. A total of 30 blood samples were collected from 30 patients with AMI (18 males and 12 females, aged 27-57 years old) who received percutaneous coronary intervention at the coronary care unit of the Second Affiliated Hospital of Harbin Medical University (Harbin, China) between May 2016 and May 2017. For patients with AMI, the following exclusion criteria applied: Previous history of myocardial infarction; having received percutaneous coronary intervention treatment; having acute heart failure upon admission; having myocardial disease, infectious pericarditis or pericardial disease; having an infectious disease, severe diabetes mellitus, malignant tumor, liver/kidney disease, pulmonary fibrosis, bone metabolic disorder, systemic immune disease or complications caused by malignant tumors; having cardiac shock. A total of 30 blood samples from 30 healthy individuals (15 males and 15 females, aged 28-61 years) admitted for general physical examination at the Second Affiliated Hospital of Harbin Medical University (Harbin, China) between May 2016 and May 2017 were recruited as the control group. None of the healthy individuals had a history of heart disease, vascular disease or other major diseases. Blood samples (5 ml) were collected from patients 6 h following the onset of AMI and frozen at -80˚C prior to the extraction of total RNA. The present study was approved by the Ethical Committee of The Second Affiliated Hospital of Harbin Medical University. All patients provided written informed consent.

Cell culture. The rat cardiomyocyte cell line H9c2 was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin mixed solution (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The cells were incubated to confluence at 37˚C with 5% CO_2 and passaged every 2-3 days (7).

Establishment of myocardial hypoxic/ischemic injury. A cell model of myocardial hypoxia/ischemia injury was established by exposing H9c2 cardiomyocytes to hypoxia for 48 h. In brief, H9c2 cells were cultured under standard conditions to reach 80% confluence, and subsequently grown in a hypoxia chamber (Thermo Fisher Scientific, Inc.) containing a gas mixture of 1% O_2, 5% CO_2 and 94% N_2 in a humidified incubator (Thermo Fisher Scientific, Inc.) at 37˚C for 48 h.

Luciferase reporter assay. TargetScan 7.1 bioinformatics software (www.targetscan.org/vert_71) was employed to identify putative targets of miR-124-3p. It was revealed that nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) repressing factor (NKRF) may be a target of miR-124-3p. To investigate this prediction, the wild-type (NKRF-WT) and mutant (NKRF-MUT) 3'-UTR of NKRF was cloned into a pmiR-RB-Report™ dual luciferase reporter gene plasmid vector (Guangzhou RiboBio Co., Ltd., Guangzhou, China). To point-mutate the miR-124-3p binding domain in the 3'-UTR of NKRF, a QuikChange Site-Directed Mutagenesis kit (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA) was used, according to the manufacturer's protocols. H9c2 cells were co-transfected with 100 ng NKRF-WT or NKRF-MUT plasmid and 50 nM miR-124-3p mimic (5'-UAA GGCACGCGGUGAAUGCC-3'; Shanghai GenePharma Co., Ltd., Shanghai, China) or mimic control (5'-UUCUCCATC GUGCCUCUAT-3'; Shanghai GenePharma Co., Ltd.) using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. Luciferase activity was determined 48 h following transfection at 37˚C using the Dual-Luciferase Assay system (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocols, and was normalized to *Renilla* luciferase activity. Experiments were repeated in triplicate.

Cell transfection. H9c2 cells were seeded in 6-well plates ($4x10^5$ cells/well). Subsequently, 100 nM negative control of miR-124-3p inhibitor (NC; 5'-CCGUACUUCGCUAGA UCA-3'; Shanghai GenePharma Co., Ltd.), 100 nM miR-124-3p inhibitor (5'-UAAGGCACGCGGUGAAUGCC-3'; Shanghai GenePharma Co., Ltd.), 1 μM control-small interfering RNA (siRNA; cat no. sc-36869; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), 1 μM NKRF-siRNA (cat no. sc-72275; Santa Cruz Biotechnology, Inc.) or 100 nM miR-124-3p inhibitor + 1 μM NKRF-siRNA was transfected into H9c2 cells using Lipofectamine 3000, according to the manufacturer's protocols. Cells were subjected to subsequent experiments 48 h following transfection at 37˚C. Transfection efficiency was determined via reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.

RT-qPCR. The expression of miR-124-3p and other genes was determined via RT-qPCR analysis. Total RNA was extracted from blood samples and cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. cDNA was synthesized using the Moloney Murine Leukemia Virus RT kit (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocols. Reaction conditions for RT were: 50˚C for 5 min and 80˚C for 2 min. qPCR was performed on the synthesized cDNAs with SYBR Green I (Applied Biosystems; Thermo Fisher Scientific, Inc.) using a CFX Connect Real-Time system (Bio-Rad Laboratories,

Inc.) according to the manufacturers' protocols. The primer sequences for qPCR were as follows: GAPDH, forward 5'-CTTTGGTATCGTGGAAGGACTC-3', reverse 5'-GTAGAGGCAGGGATGATGTTCT-3'; U6, forward 5'-GCTTCGGCAGCACATATACTAAAAT-3', reverse 5'-CGCTTCACGAATTTGCGTGTCAT-3'; miR-124-3p, forward 5'-GCTAAGGCACGCGGTG-3', reverse 5'-GTGCAGGGTCCGAGGT-3'; tumor necrosis factor-α (TNF-α), forward 5'-GAACTGGCAGAAGAGGCACT-3', reverse 5'-GGTCTGGGCCATAGAACTGA-3'; interleukin (IL)-1β, forward 5'-TGTGAAATGCCACCTTTTGA-3', reverse 5'-TGAGTGATACTGCCTGCCTG-3'; IL-6, forward 5'-CCGGAGAGGAGACTTCACAG-3', reverse 5'-CAGAATTGCCATTGCACA-3'; and NKRF, forward 5'-TATTGATATTGGGGAGATGCC-3' and reverse 5'-GGATCTTCCTGTCTTTCATCT-3'. Thermocycling was conducted as follows: 95˚C for 5 min, followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing/elongation at 60˚C for 30 sec. GAPDH (for mRNA) and U6 (for miR-124-3p) were used as internal controls. The $2^{-\Delta\Delta Cq}$ method (23) was used to determine the relative gene expression. Experiments were repeated in triplicate.

Western blot analysis. To measure the protein levels of NKRF, phosphorylated (p)-p65, B-cell lymphoma 2 (Bcl-2), and pro-caspases and cleaved caspases 3 and 9, western blot analysis was performed. Total protein was extracted from H9c2 cells using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China), according to the manufacturer's protocols. The concentration of protein was determined using a Bicinchoninic Acid Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Lysate samples (25 μg/lane) were separated via 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes prior to blocking with 5% skimmed milk at room temperature for 1.5 h. The membranes were incubated overnight at 4˚C with primary antibodies against: NKRF (1:1,000; cat. no. ab168829; Abcam, Cambridge, MA, USA), p-p65 (1:1,000; cat no. 3033; Cell Signaling Technology, Inc., Danvers, MA, USA), p65 (1:1,000; cat no. 8242; Cell Signaling Technology, Inc.), Bcl-2 (1:1,000; cat no. 4223; Cell Signaling Technology, Inc.), cleaved caspase 3 (1:1,000; cat no. 9664; Cell Signaling Technology, Inc.), cleaved caspase 9 (1:1,000; cat no. 9505; Cell Signaling Technology, Inc.), pro-caspase 3 (1:1,000; cat. no. ab32150; Abcam), pro-caspase 9 (1:1,000; cat. no. ab135544; Abcam) and β-actin (1:1,000; cat. no. 4970; Cell Signaling Technology, Inc.). Membranes were subsequently incubated at room temperature for 4 h with the horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibodies (cat no. 7074; 1:2,000; Cell Signaling Technology, Inc.). Protein bands were visualized using a SuperSignal West Dura Extended Duration Substrate enhanced chemiluminescence detection system (Pierce; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. Protein expression was quantified using Gel-Pro Analyzer Version 6.3 densitometry software (Media Cybernetics, Inc., Rockville, MD, USA). Experiments were repeated in triplicate.

MTT assay. The viability of cells was determined using an MTT assay. Briefly, H9c2 cells were seeded into 96-well culture plates and grown at 37˚C for 24 h. Subsequently, 5 mg/ml MTT solution (Amresco, LLC, Solon, OH, USA) was added to each culture well, and cells were incubated for a further 4 h. Subsequently, 150 μl DMSO was used to dissolve the purple formazan and the absorbance was detected at 490 nm using a Synergy™ 2 Multi-Mode microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Experiments were repeated in triplicate.

Apoptosis assay. To determine the apoptosis of cells, an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (cat no. 70-AP101-100; Hangzhou MultiSciences Biotech, Co., Ltd., Hangzhou, China) was used. Briefly, H9c2 cells were washed with PBS three times and fixed in 70% ethanol at 4˚C overnight, and stained with PI/Annexin V-FITC, according to the manufacturer's protocols. A flow cytometer was used (BD Biosciences, Franklin Lakes, NJ, USA) to analyze cell apoptosis, and the apoptosis rate was calculated as the total percentage of cells in the right-side quadrants (early + late apoptotic cells) using FlowJo software (version 7.6.1; FlowJo LLC, Ashland, OR, USA) was used for data analysis. Experiments were repeated in triplicate.

ELISA. An ELISA was performed to determine the levels of TNF-α, IL-1β and IL-6 in cell supernatants. Briefly, H9c2 cells were transfected with NC, miR-124-3p inhibitor, or miR-124-3p inhibitor + NKRF-siRNA for 2 h and then subjected to hypoxia for 48 h. Cell supernatants were harvested via centrifugation (1,000 x g, 15 min, 4˚C). The expression levels of IL-1β (cat no. PI305; Beyotime Institute of Biotechnology), TNF-α (cat no. PI518; Beyotime Institute of Biotechnology), and IL-6 (cat no. PI330; Beyotime Institute of Biotechnology) were detected using ELISA kits, according to the manufacturer's protocols. Experiments were repeated in triplicate.

Statistical analysis. Data are presented as the mean ± standard deviation of at least three experiments. All data were analyzed using SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA). Comparisons between groups were performed using Student's t-tests or one-way analyses of variance followed by a Tukey's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-124-3p is upregulated during AMI. RT-qPCR analysis was performed to investigate the expression of miR-124-3p in AMI. As presented in Fig. 1A, the expression levels of miR-124-3p were significantly increased in the blood of patients with AMI compared with healthy controls. Additionally, it was revealed that the expression of miR-124-3p in the cardiomyocyte cell line H9c2 was significantly upregulated following exposure to hypoxic conditions for 48 h compared with the control (Fig. 1B).

NKRF is a target of miR-124-3p. TargetScan was employed to predict potential targets of miR-124-3p. Various target genes of miR-124-3p, including NKRF (Fig. 2A). Previous studies indicated that NKRF is a silencer protein that binds

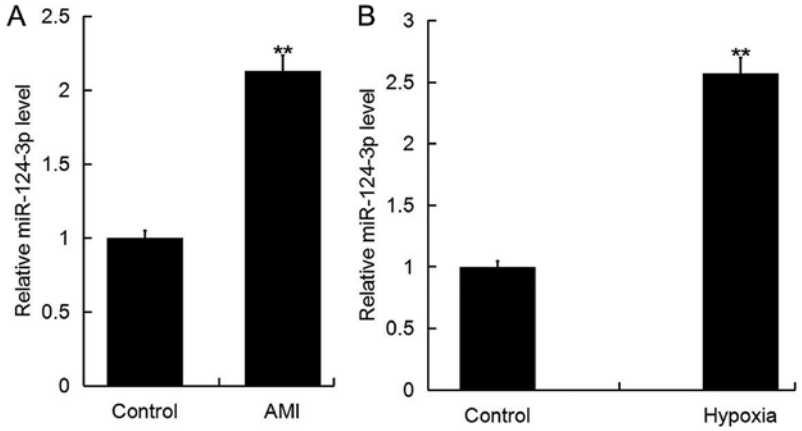

Figure 1. Expression of miR-124-3p in AMI. (A) Expression of miR-124-3p in the blood of patients with AMI and healthy controls, as determined by RT-qPCR analysis. (B) Expression of miR-124-3p in H9c2 cardiomyocytes exposed to hypoxic or control conditions for 48 h, as determined by RT-qPCR analysis. Data are presented as the mean ± standard deviation of experiments repeated in triplicate. **P<0.01 vs. control. AMI, acute myocardial injury; miR-124-3p, microRNA-124-3p; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

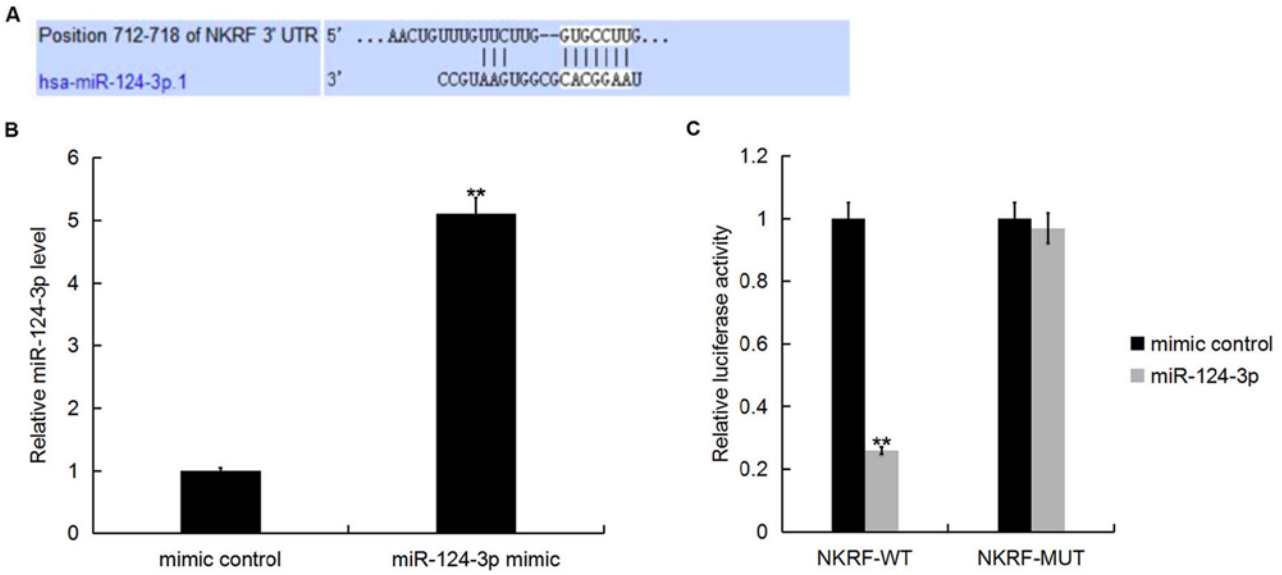

Figure 2. NKRF is a target of miR-124-3p. (A) Binding site of miR-124-3p in the 3'-UTR of NKRF as predicted by TargetScan. (B) Expression of miR-124-3p in H9c2 cardiomyocytes transfected with mimic control or miR-124-3p mimic. (C) Luciferase activity of a reporter containing NKRF-WT or NKRF-MUT in H9c2 cells following co-transfection with mimic control or miR-124-3p mimic. Data are presented as the mean ± standard deviation of three independent experiments. **P<0.01 vs. mimic control. miR-124-3p, microRNA-124-3p; NKRF, nuclear factor κ-light-chain-enhancer of activated B cells repressing factor; 3'-UTR, 3'-untranslated region; NKRF-WT, wild-type 3'-UTR of NKRF; NKRF-MUT, mutant 3'-UTR of NKRF.

negative regulatory elements (NRE) to repress the transcription of various NF-κB-regulated genes (24). Therefore, NKRF serves important roles in inflammation responses and apoptosis; however, the involvement of this gene in AMI remains unclear. Thus, NKRF was selected for further investigation in the present study.

To determine whether miR-124-3p directly modulates the expression of NKRF via interactions with potential binding sites in the 3'-UTR, a luciferase reporter assay was performed. Transfection of H9c2 cells with miR-124-3p mimic upregulated the expression of miR-124-3p compared with the mimic control (Fig. 2B). Furthermore, co-transfection of H9c2 cells with miR-124-3p mimic and NKRF-WT plasmid significantly decreased the luciferase activity compared with co-transfection with miR-124-3p mimic and NKRF-MUT plasmid (Fig. 2C). The results indicated that miR-124-3p directly targeted NKRF mRNA.

miR-124-3p inhibition suppresses the hypoxia-induced inhibition of the viability of H9c2 cells. To investigate the effects of miR-124-3p in AMI, H9c2 cells were transfected with NC, miR-124-3p inhibitor, control-siRNA, NKRF-siRNA or miR-124-3p inhibitor + NKRF-siRNA, and the transfection efficiency was determined 48 h following transfection. Transfection with miR-124-3p inhibitor significantly downregulated the expression of miR-124-3p compared with the control (Fig. 3A), whereas NKRF-siRNA significantly downregulated the expression of NKRF mRNA (Fig. 3B) and protein (Fig. 3C and D) in H9c2 cells compared with control-siRNA. It was further demonstrated that miR-124-3p

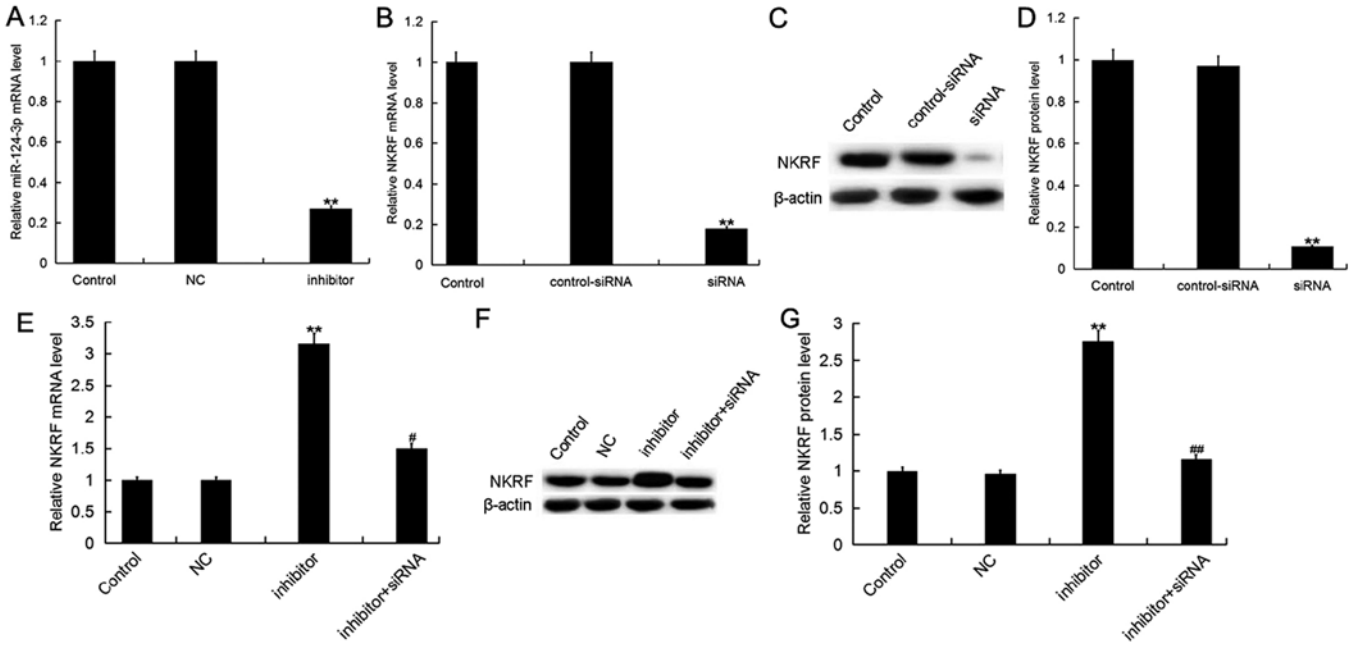

Figure 3. Downregulation of miR-124-3p promotes NKRF expression in H9c2 cells. (A) Expression of miR-124-3p in H9c2 cardiomyocytes transfected with inhibitor or NC. Expression of NKRF (B) mRNA or protein, as measured by (C) western blotting and (D) quantified, in H9c2 cells transfected with NKRF-siRNA or control-siRNA. Expression of NKRF (E) mRNA or protein, as measured by (F) western blotting and (G) quantified, in H9c2 cells transfected with inhibitor in the presence and absence of NKRF-siRNA. Data are presented as the mean ± standard deviation of three independent experiments. **P<0.01 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. inhibitor. miR-124-3p, microRNA-124-3p; inhibitor, miR-124-3p inhibitor; NC, negative control; NKRF, nuclear factor κ-light-chain-enhancer of activated B cells repressing factor; siRNA, small interfering RNA.

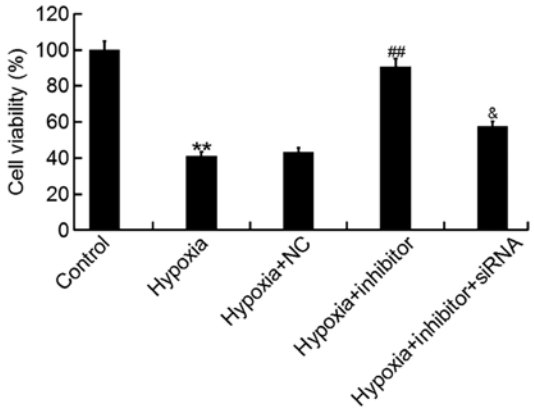

Figure 4. Effects of microRNA-124-3p expression on the viability of H9c2 cells. MTT assays were performed to determine the viability of H9c2 cardiomyocytes following exposure to hypoxic or control conditions for 48 h, and transfection with NC, inhibitor or inhibitor + NKRF-siRNA. Data are presented as the mean ± standard deviation of three independent experiments. **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. hypoxia; $^{\&}$P<0.05 vs. hypoxia + inhibitor. Inhibitor, miR-124-3p inhibitor; NC, negative control; NKRF, nuclear factor κ-light-chain-enhancer of activated B cells repressing factor; siRNA, small interfering RNA.

inhibitor significantly increased the expression levels of NKRF at the mRNA (Fig. 3E) and protein (Fig. 3F and G) levels; these effects were eliminated by transfection with NKRF-siRNA.

An MTT assay was performed to determine the effects of miR-124-3p on the viability of H9c2 cells following hypoxia. It was revealed that miR-124-3p inhibitor significantly attenuated the hypoxia-induced decrease in the viability of H9c2 cells, whereas co-transfection with inhibitor and NKRF-siRNA eliminated the effects of the inhibitor (Fig. 4).

miR-124-3p inhibition suppresses the hypoxia-induced apoptosis of H9c2 cells. The effects of miR-124-3p on the apoptosis of H9c2 cells were determined via flow cytometry. It was demonstrated that hypoxia significantly promoted H9c2 cell apoptosis, whereas transfection with miR-124-3p inhibitor attenuated the effects of hypoxia (Fig. 5A and B). Furthermore, co-transfection with NKRF-siRNA significantly eliminated the effects of miR-124-3p inhibitor on the apoptosis of H9c2 cells.

To further investigate the antiapoptotic effects of miR-124-3p inhibitor, the expression of apoptosis-associated proteins was determined, including Bcl-2, cleaved caspase 3, cleaved caspase 9, procaspase 3 and procaspase 9. It was observed that the protein expression levels of Bcl-2, procaspase 3 and procaspase 9 were markedly decreased in H9c2 cells following hypoxia, and those of cleaved caspase 3 and cleaved caspase 9 were significantly increased in H9c2 cells treated with hypoxia, compared with the control; transfection with miR-124-3p inhibitor reversed the hypoxia-induced effects (Fig. 5C). Furthermore, the effects of miR-124-3p inhibitor on the expression of Bcl-2, cleaved caspase 3, cleaved caspase 9, procaspase 3 and procaspase 9 in H9c2 cells were eliminated by the silencing of NKRF.

miR-124-3p inhibition suppresses hypoxia-induced inflammatory responses in H9c2 cells. As inflammation serves important roles in the development of AMI, the effects of miR-124-3p on inflammatory responses during AMI were investigated. As presented in Fig. 6, the protein and mRNA expression levels of TNF-α, IL-1β and IL-6 were significantly increased following hypoxia; however, transfection with miR-124-3p inhibitor significantly inhibited the hypoxia-induced

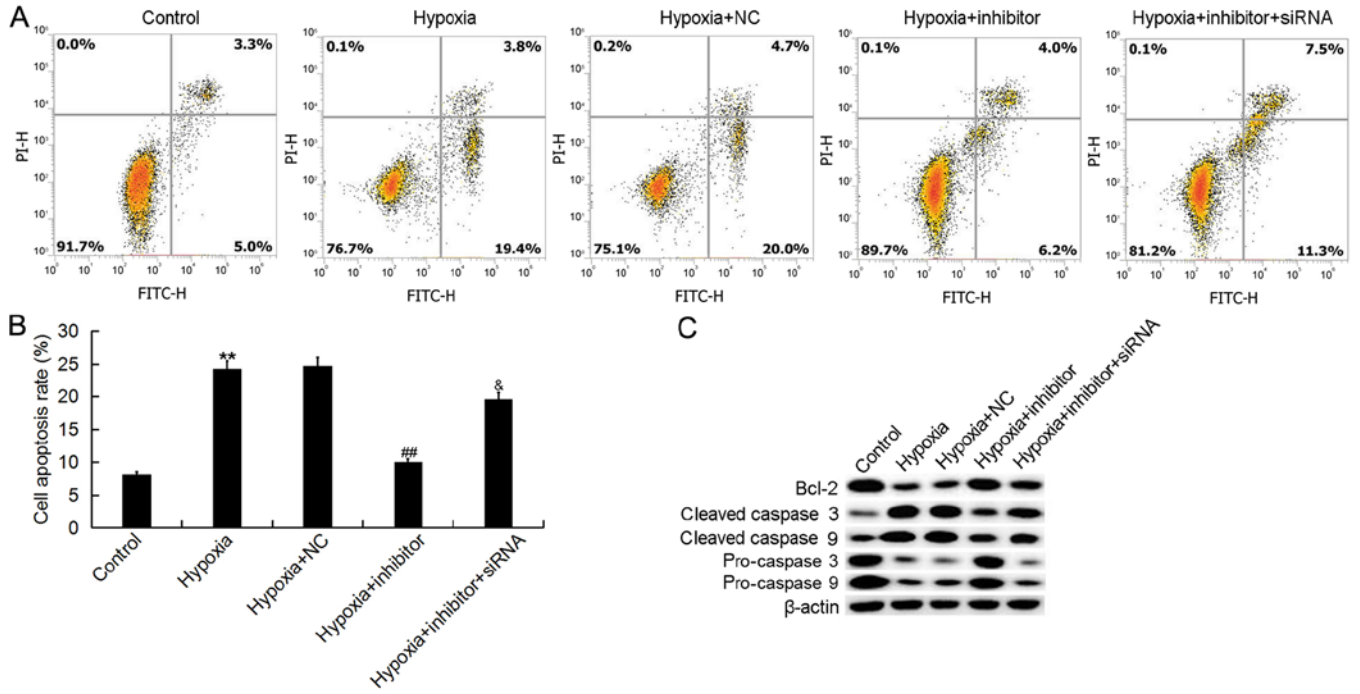

Figure 5. Effects of microRNA-124-3p expression on the apoptosis of H9c2 cells. (A) Flow cytometry was performed to determine the apoptosis of H9c2 cardiomyocytes following exposure to hypoxic or control conditions for 48 h, and transfection with NC, inhibitor or inhibitor + NKRF-siRNA. (B) Quantification of the apoptosis of H9c2 cells. (C) Expression of Bcl-2, and cleaved and procaspases 3 and 9 following the aforementioned treatments. Data are presented as the mean ± standard deviation of three independent experiments. **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. hypoxia; $^{\&}$P<0.05 vs. hypoxia + inhibitor. Bcl-2, B-cell lymphoma 2; FITC, fluorescein isothiocyanate; Inhibitor, miR-124-3p inhibitor; NC, negative control; NKRF, nuclear factor κ-light-chain-enhancer of activated B cells repressing factor; PI, propidium iodide; siRNA, small interfering RNA.

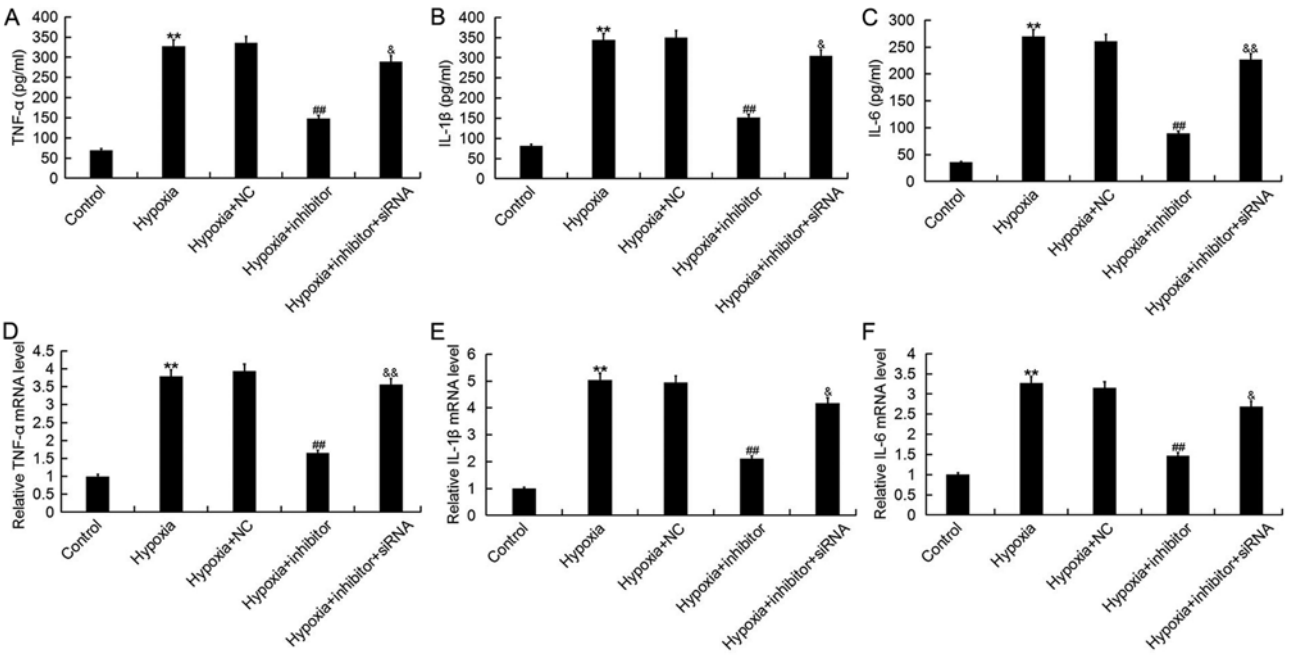

Figure 6. Effects of microRNA-124-3p expression on the levels of TNF-α, IL-1β and IL-6 in H9c2 cells. Expression of (A) TNF-α, (B) IL-1β and (C) IL-6 protein in H9c2 cardiomyocytes following exposure to hypoxic or control conditions for 48 h, and transfection with NC, inhibitor or inhibitor + NKRF-siRNA, as determined by ELISA. Expression of (D) TNF-α, (E) IL-1β and (F) IL-6 mRNA in H9c2 cells following the aforementioned treatments, as determined by reverse transcription-quantitative polymerase chain reaction analysis. Data are presented as the mean ± standard deviation of three independent experiments. **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. hypoxia; $^{\&}$P<0.05, $^{\&\&}$P<0.01 vs. hypoxia + inhibitor. IL, interleukin; Inhibitor, miR-124-3p inhibitor; NC, negative control; NKRF, nuclear factor κ-light-chain-enhancer of activated B cells repressing factor; siRNA, short interfering RNA; TNF-α, tumor necrosis factor-α.

upregulation of inflammatory cytokines, effects which were eliminated following co-transfection with NKRF-siRNA. The results indicated that miR-124-3p inhibitor suppressed hypoxia-induced inflammatory responses in cardiomyocytes.

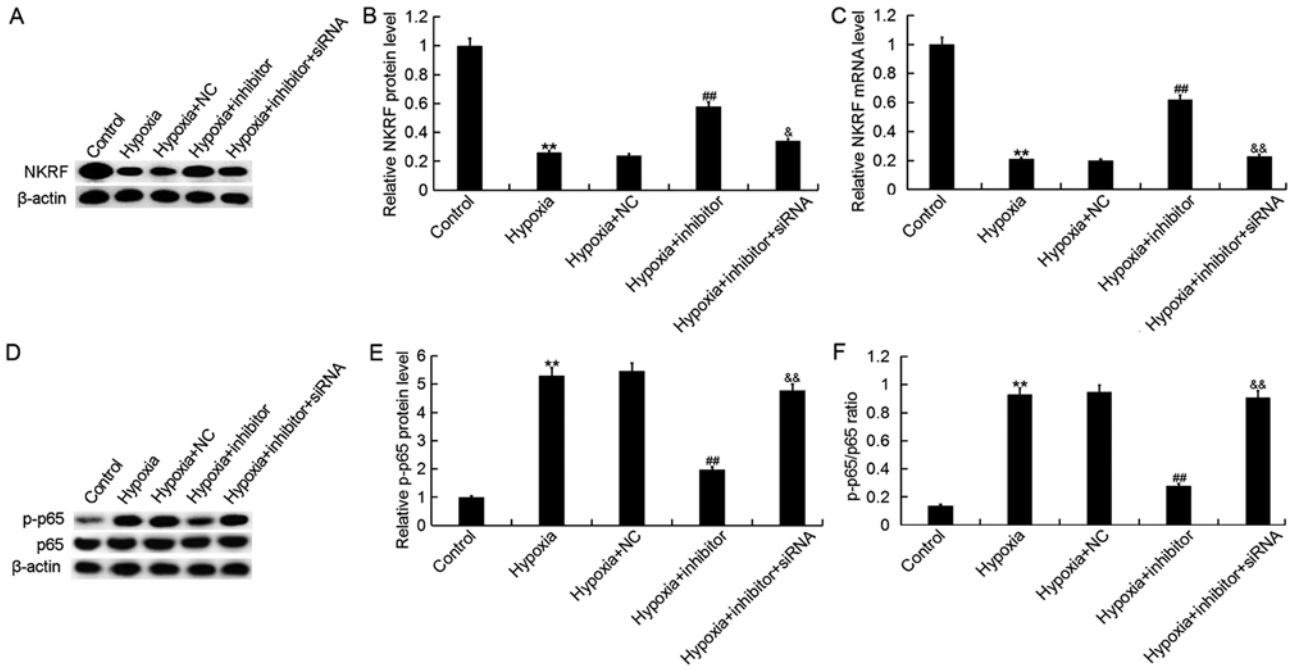

Figure 7. Effects of miR-124-3p expression on the NF-κB pathway in H9c2 cells. Expression of NKRF protein, assessed by (A) western blotting and (B) quantification, and (C) mRNA in H9c2 cardiomyocytes following exposure to hypoxic or control conditions for 48 h, and transfection with NC, inhibitor or inhibitor + NKRF-siRNA, as determined by western blot and reverse transcription-quantitative polymerase chain reaction analyses, respectively. (D) Expression of NF-κB p65 and p-p65 in H9c2 cells following the aforementioned treatments, as determined via western blotting. Expression of p-p65 normalized to (E) the internal control and (F) p65 expression. Data are presented as the mean ± standard deviation of three independent experiments. **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. hypoxia; $^{\&}$P<0.05, $^{\&\&}$P<0.01 vs. hypoxia + inhibitor. Inhibitor, miR-124-3p inhibitor; NC, negative control; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; NKRF, NF-κB repressing factor; p, phosphorylated; siRNA, small interfering RNA.

miR-124-3p inhibitor suppresses the hypoxia-induced activation of NF-κB signaling in H9c2 cells. To investigate the mechanisms underlying the effects of miR-124-3p inhibitor on the cardiomyocyte cell line H9c2, the activity of the NF-κB pathway was analyzed. As presented in Fig. 7, hypoxia significantly downregulated the expression of NKRF (Fig. 7A-C) and promoted the phosphorylation of NF-κB p65 in H9c2 cells (Fig. 7D-F); transfection with miR-124-3p inhibitor significantly attenuated the effects of hypoxia on NKRF expression and p65 phosphorylation. Additionally, it was demonstrated that NKRF-siRNA significantly eliminated the effects of miR-124-3p inhibitor on NKRF and p-p65 levels in H9c2 cells. The expression of p65 protein in H9c2 cells was not notably different across the various groups.

Discussion

In the present study, it was demonstrated that expression of miR-124-3p was significantly increased during AMI. Downregulation of miR-124-3p opposed the effects of hypoxia on the viability, apoptosis and inflammatory responses of H9c2 cells by targeting NKRF. In addition, the results suggested that miR-124-3p downregulation inhibited the hypoxia-induced activation of the NF-κB pathway. The findings of the present study indicated that miR-124-3p inhibitor protected H9c2 cardiomyocytes against hypoxia, and that miR-124-3p may be a promising therapeutic target in the treatment of AMI.

AMI is an acute necrotic myocardial infarction induced by persistent and severe ischemia, and is one of the most common cardiovascular diseases globally (25). At present, there are widely available treatments; however, the prevalence of cardiovascular diseases, mortality and treatment costs continue to increase in developed and developing countries (26). To effectively reduce the occurrence and development of AMI, it is necessary to identify novel targets and methods for the diagnosis and treatment of AMI.

miRNAs have received increasing attention in various research fields, such as cancer and cardiovascular diseases (27,28). The abnormal expression of miRNAs has been closely associated with the pathophysiological processes of AMI (20,21). A previous study reported the upregulation of miR-124 in the blood of patients with AMI (22); however, the expression and role of miR-124-3p in the development of AMI remain unclear. Therefore, the present study was conducted.

The levels of miR-124-3p during AMI were determined; it was observed that miR-124-3p levels were significantly increased in the blood of patients with AMI and hypoxia-treated H9c2 cells, indicating the potential role of miR-124-3p in AMI. It was subsequently identified that NKRF was a direct target of miR-124-3p.

Persistent inflammatory responses and the necrosis of ischemic tissue are two prominent features that are mutually reinforced during AMI-induced heart damage, eventually leading to heart failure (29). Inflammation is the principal pathological process that occurs during early MI; inflammatory factors serve important roles in ventricular remodeling and the progression of heart failure (30,31). Apoptosis and necrosis are the two notable events during the development of AMI (32,33). Therefore, determination of the molecular mechanisms underlying the apoptosis and inflammatory responses

of cardiomyocytes is required to develop effective treatment strategies for ischemic heart disease. Numerous studies have reported that miR-124-3p was involved in the development of various diseases by regulating the proliferation and apoptosis of cells (34-37). miR-124-3p has also been reported to contribute to the regulation of inflammatory responses (38). A recent study demonstrated that the long non-coding RNA taurine upregulated gene 1 alleviated hypoxia-induced injury (as determined by an increase in the viability and a decrease in the apoptosis of cells) by targeting miR-124 in H9c2 cells, and that miR-124 promoted hypoxia-induced effects in H9c2 cells by regulating the expression of hydrogen peroxide-inducible clone-5 (39). The present study reported that hypoxia-induced reductions in the viability and increases in the apoptosis of H9c2 cells were opposed by miR-124-3p downregulation. Furthermore, the hypoxia-induced upregulation of TNF-α, IL-1β and IL-6 was inhibited by miR-124-3p downregulation. Conversely, silencing NKRF eliminated all the effects of miR-124-3p inhibitor on H9c2 cells.

It was revealed that NKRF was a direct target of miR-124-3p. NKRF is a silencer protein that binds NREs to suppress the basal transcription of NF-κB-regulated genes (40). Therefore, the effects of miR-124-3p on the NF-κB pathway were investigated in cardiomyocytes, and the findings suggested that miR-124-3p inhibitor inhibited the hypoxia-induced activation of the NF-κB pathway in H9c2 cells; this inhibition was eliminated by NKRF silencing.

In conclusion, it was demonstrated that the expression of miR-124-3p was abnormally high in AMI, and its inhibition suppresses inflammatory responses and apoptosis in a cell model of AMI in an NKRF-dependent manner. miR-124-3p may be a novel therapeutic target in the treatment of AMI; however, as this is a preliminary study into the role of miR-124-3p in AMI, further investigation is required. For example, the expression of NKRF in the blood of patients with AMI should be determined. Furthermore, as a cellular model of AMI is notably different to the human disease, *in vivo* and clinical studies are required to demonstrate the role of miR-124-3p in AMI and supporting the findings observed *in vitro*.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GH designed the current study, collected and analyzed the data, performed statistical analysis, searched the literature, and prepared the manuscript. LM, FD and XH contributed to data collection and data interpretation. SL and HS contributed to statistical analyses and interpreted the data.

Ethics approval and consent to participate

The present study was approved by the Ethical Committee of The Second Affiliated Hospital of Harbin Medical University. All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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