

# Effects of nuclear respiratory factor-1 on apoptosis and mitochondrial dysfunction induced by cobalt chloride in H9C2 cells

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**Abstract.** Hypoxia-induced apoptosis occurs in various diseases. Cobalt chloride (CoCl<sub>2</sub>) is a hypoxia mimic agent that is frequently used in studies investigating the mechanisms of hypoxia. Nuclear respiratory factor-1 (NRF-1) is a transcription factor with an important role in the expression of mitochondrial respiratory and mitochondria-associated genes. However, few studies have evaluated the effects of NRF-1 on apoptosis, particularly with regard to damage caused by CoCl<sub>2</sub>. In the present study, the role of NRF-1 in mediating CoCl<sub>2</sub>-induced apoptosis was investigated using cell viability analysis, flow cytometry, fluorescence imaging, western blotting analysis, energy metabolism analysis and reverse transcription-quantitative polymerase chain reaction. The present results revealed that the apoptosis caused by CoCl<sub>2</sub> could be alleviated by NRF-1. Furthermore, overexpression of NRF-1 increased the expression of *B-cell lymphoma-2*, *hypoxia inducible factor-1α* and *NRF-2*. Also, cell damage induced by CoCl<sub>2</sub> may be associated with depolarization of mitochondrial membrane potential, and NRF-1 suppressed this effect. Notably, the oxygen consumption rate (OCR) was reduced in CoCl<sub>2</sub>-treated cells, whereas overexpression of NRF-1 enhanced the OCR,

suggesting that NRF-1 had protective effects. In summary, the present study demonstrated that NRF-1 protected against CoCl<sub>2</sub>-induced apoptosis, potentially by strengthening mitochondrial function to resist CoCl<sub>2</sub>-induced damage to H9C2 cells. The results of the present study provide a possible way for the investigation of myocardial diseases.

## Introduction

Dysfunction caused by hypoxia is an important component of numerous diseases (1). Tissue hypoxia is induced by decreased blood flow, arterial blood oxygen partial pressure and interrupted blood flow (2). When oxygen supply is insufficient or the balance between O<sub>2</sub> demand and supply is disrupted, distant organs can be damaged, and neuronal injury (2) or microcirculation disturbance in perfusion organs, such as the liver, kidneys and gastrointestinal tract (3), may occur. The heart is at the center of blood and oxygen supply to all organs. However, hypoxia is also a major contributor to cardiomyocyte pathophysiology, with a role in diseases including congenital heart disease, myocardial infarction (4-6) and ischemic heart disease (7,8).

The metabolic center of oxygen consumption is the mitochondria, which can produce up to 90% of the adenosine triphosphate (ATP) required for cell function (9-12). Previous studies have demonstrated that the mitochondria act as a power source for the cell and as an effector of other critical events, including apoptosis and cell death (13,14). Therefore, it is important to understand their function, particularly with regard to metabolism. In contrast to other subcellular structures that are regulated by karyogenes, mitochondria contain their own transcription system. Mitochondrial DNA (mtDNA) is a double-stranded circular DNA containing 37 genes and encoding 13 polypeptides that are involved in the synthesis of ATP (15,16). Although ~1,500 proteins participate in the regulation of mitochondrial structure and function, because of the limited coding capacity of mtDNA, <1% of these proteins are encoded by mitochondria, and the remaining proteins are encoded by nuclear genes (17-19). Therefore, the biological processes of mitochondria are regulated by both mitochondrial and nuclear genes.

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Nuclear respiratory factor-1 (NRF-1), which was initially discovered during the investigation of cytochrome *c* transcriptional activation (20), is essential for early embryogenesis in mammals, and loss of NRF-1 results in a peri-implantation lethal phenotype. Furthermore, NRF-1<sup>-/-</sup> blastocysts exhibited decreased mtDNA amounts (21). NRF-1 also serves an important role in the integration of nuclear and mitochondrial interactions (20,22-24). For example, NRF-1 mediates the transcription of mtDNA by affecting the promoter region of mitochondrial transcription factor A (mtTFA; also termed Tfam) (25), thus altering mitochondrial biogenesis (26-28). Nuclear factor (NF)- $\kappa$ B can regulate the *NRF-1* gene directly via the lipopolysaccharide-receptor pathway, leading to increased mitochondrial mRNA transcription and enrichment of mtDNA copy number (29). Furthermore, in aerobic cardiac cells, NRF-1 is associated with the transcriptional control of complex II and prevention of pseudo-hypoxic gene expression (30).

Cobalt chloride (CoCl<sub>2</sub>) is often used as a hypoxia mimic agent *in vivo* and *in vitro* (31,32) and it has been demonstrated to activate hypoxia-associated signals, such as stabilizing hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) (33,34). HIF-1 $\alpha$  can be hydroxylated and then ubiquitinated for degradation by the proteasome in normoxic conditions (35-37); however, under hypoxic conditions or in the presence of low oxygen concentrations, the  $\alpha$  subunit is not hydroxylated, allowing HIF-1 $\alpha$  to enter the nucleus inducing the transcription of certain hypoxia response elements (38-40).

Therefore, in the present study, it was aimed to further elucidate the role of NRF-1 in hypoxia. To this end, the effects of NRF-1 overexpression in H9C2 cardiomyoblasts on CoCl<sub>2</sub>-stimulated hypoxia were investigated.

## Materials and methods

**Materials.** The lentiviral expression vector pLenti6.3-NRF1-IRES2-EGFP and lentiviral packaging plasmids (pLP1, pLP2 and pLP/VSVG) were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). H9C2 cells were purchased from cell bank of the Chinese Academy of Sciences (Shanghai, China). Plasmid extraction and purification kits purchased from Axygen (Corning Incorporated, Corning, NY, USA). TRIzol reagent, 0.25% Trypsin, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and 293T cells were purchased from Invitrogen (Thermo Fisher Scientific, Inc.). The Cell Counting Kit-8 (CCK-8) was purchased from TransGen Biotech (Beijing, China). Hoechst 33342 was purchased from Beyotime Institute of Technology (Haimen, China). TransScript Reverse Transcriptase and qPCR SuperMix were purchased from TransGen Biotech.

**NRF-1 transfection.** 293T packaging cells (1 $\times$ 10<sup>7</sup>) were plated in 10-cm plates before transfection. pLenti6.3-NRF1-IRES2-EGFP plasmids (3  $\mu$ g) and 9  $\mu$ g packaging plasmids (3  $\mu$ g pLP1, 3  $\mu$ g pLP2 and 3  $\mu$ g pLP/VSVG) were co-transfected into the 293T cells using Lipofectamine<sup>®</sup> 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and inoculated in a 10 cm culture dish before transfection. Virus-containing supernatant was isolated under 50,000  $\times$  g

at 4°C and collected after 2 h. Virus was added to the H9C2 cells (1 $\times$ 10<sup>5</sup>/ml) in the presence of 8  $\mu$ g/ml polybrene (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Following transfection for 48 h, the target cells were subjected to 1  $\mu$ g/ml puromycin for selection. The transfected cells were designated as NRF1-transfected H9C2 (NRF1-H9C2) cells and empty virus-transfected as pLenti-H9C2 cells.

**Cell culture and treatment.** NRF1-H9C2 or pLenti-H9C2 cells (5 $\times$ 10<sup>6</sup>) were cultured in 10-cm culture plates in DMEM supplemented with 10% FBS and 2 mM glutamine and incubated in a humidified incubator with an atmosphere containing 5% CO<sub>2</sub> and 21% O<sub>2</sub> at 37°C. Chemical hypoxia was induced by adding the hypoxia-mimetic agent CoCl<sub>2</sub> (Sigma-Aldrich; Merck KGaA) at 200 or 400  $\mu$ M, and cells were then incubated for 6 or 24 h (41,42).

**Determination of cell viability.** 5 $\times$ 10<sup>4</sup> NRF1-H9C2 and pLenti-H9C2 cells (5 $\times$ 10<sup>6</sup>) were seeded in 96-well plates and treated with 200 or 400  $\mu$ M CoCl<sub>2</sub> for 6 or 24 h. Subsequently, 10  $\mu$ l CCK-8 reagent was added to each well, and the plates were incubated at 37°C for 3 h. Absorbance was measured at 450 nm using a microplate reader. The cell viability (%) relative to the control was calculated as follows: Relative cell viability (%) = optical density (OD) sample/OD control  $\times$  100. Each group was analyzed using five wells, and the experiment was repeated at least three times.

**Analysis of mitochondrial membrane potential (MMP).** Cells (5 $\times$ 10<sup>5</sup>) were seeded in 6-well plates and the cells were stained with 2.5 nM tetramethylrhodamine ethyl ester (Sigma-Aldrich; Merck KGaA) for 30 min at 37°C (43), then washed twice with PBS and analyzed using an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and BD Accuri C6 Software (version 1.0; BD Biosciences).

**Analysis of apoptosis by Hoechst 33342 staining.** Cells (2 $\times$ 10<sup>5</sup>) were seeded in 24-well plates and propagated to ~80% confluence, then washed with PBS twice, stained with Hoechst 33342 (50  $\mu$ g/ml) at 37°C, and incubated in the dark. After 20 min, the nuclear morphology of the cells was observed to determine the occurrence of apoptosis using a fluorescence microscope.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.** Total RNA was extracted using TRIzol reagent in accordance with the manufacturer's protocol. cDNA was synthesized from total RNA using Transcript Reverse Transcriptase (Transgene Biotech; the reaction system is presented as Table I) then incubated for 30 min at 42°C and heated at 85°C for 5 sec. Fluorescent qPCR was performed using Transcript Green Two-Step qRT-PCR Super Mix (Transgene Biotech). Specific primers were used to amplify *NRF-1*, *NRF-2*, *mtTFA*, *HIF-1 $\alpha$* , mitochondrial single stranded DNA binding protein (*mtSSB*), Bcl-associated X protein (*Bax*), B-cell lymphoma-2 (*Bcl-2*), and *GAPDH*, as presented in Table II. All fluorescent qPCR assays were performed in duplicate 20- $\mu$ l reactions, and detection was carried out using a Light Cycler 2 real-time PCR machine (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The thermocycling conditions were: 95°C for 2 min followed by 35 cycles of amplification

Table I. cDNA reaction system.

Component	Volume
Total RNA	1 $\mu$ l (50 ng-5 $\mu$ g)
Oligo(dT) Primer	1 $\mu$ l
10 mM dNTPs	1 $\mu$ l
5xBuffer	4 $\mu$ l
Ribonuclease Inhibitor	0.5 $\mu$ l
TransScript RT	1 $\mu$ l
RNase-free Water	11.5 $\mu$ l
Total volume	20 $\mu$ l

(95°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec). The data was quantified by 2<sup>- $\Delta\Delta$ C<sub>q</sub></sup> method (44).

**Western blotting.** Cells were lysed using cell lysis buffer (Beyotime Institute of Biotechnology). Homogenate protein concentrations were measured using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Cell lysates (50  $\mu$ g) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% gels and transferred onto polyvinylidene difluoride membranes (EMD Millipore). The membranes were blocked with 5% nonfat milk in PBS containing 0.1% Tween-20 for 2 h at room temperature and then incubated overnight at 4°C with anti-NRF1 (1:1,000; ab175932), anti-Bax (1:1,000; ab32503), anti-Bcl-2 (1:500; ab59348), anti-poly(ADP-ribose) polymerase 1 (PARP; 1:500; ab32064) and anti- $\beta$ -actin (1:2,000; ab49900); all from Abcam (Cambridge, UK)]. Subsequently, the membranes were incubated with horseradish peroxidase-linked secondary anti-rabbit (1:5,000; BA1054; Boster Biological Technology, Pleasanton, CA, USA) at room temperature for 2 h. Antibody-bound protein was detected using a chemiluminescent detection kit (Super Signal West Dura; Thermo Fisher Scientific, Inc.), exposed to X-ray film, and densitometry was quantified by Image Lab version 5.1 (Bio-Rad Laboratories, Inc.).

**Annexin V-allophycocyanin (APC)/7-aminoactinomycin D (7-AAD) staining.** Cells were seeded in 24-well plates (2x10<sup>5</sup> per well) and cultured with medium containing 400  $\mu$ M CoCl<sub>2</sub> for 6 h. Apoptotic cells were measured by flow cytometry using Annexin V-APC and 7-AAD Apoptosis Detection reagent (BD Biosciences). Briefly, cell were digested, collected, washed and incubated at room temperature for 10 min with 1X binding buffer containing Annexin V-conjugated APC and 7-AAD. Annexin V-APC staining was used to detect exposure of phosphatidylserine. 7-AAD is able to penetrate the cells when cell apoptosis and cell death occurs. Annexin V-APC was used to detect early apoptotic cells, and Annexin V-APC and 7-AAD were used to detect necrotic cells or/and nonviable apoptotic cells. Cells were detected by BD Accuri C6 Flow Cytometer and analyzed by BD Accuri™ C6 Software version 1.0 (BD Biosciences).

**Detection of energy metabolism.** An XF<sup>®</sup>24 Analyzer (Seahorse Bioscience; Agilent Technologies, Inc., Santa

Table II. Primers used for reverse transcription-quantitative polymerase chain reaction.

Gene	Direction	Sequence (5'-3')
NRF-1	Sense	CCACGTTGGATGAGTACACG
	Antisense	CTGAGCCTGGGTCATTTTGT
NRF-2	Sense	CCGCTACACCGACTACGATT
	Antisense	ACCTTCATCACCAACCCAAG
mtTFA	Sense	GCTTCCAGGAGGCTAAGGAT
	Antisense	CCCAATCCCAATGACAACCTC
HIF-1 $\alpha$	Sense	TGCTGATTTGTGAACCCATT
	Antisense	TCTGGCTCATAACCCATCAA
mtSSB	Sense	AGCCAGCAGTTTGGTTCTTG
	Antisense	CCACTTTGCCTTCCACAAAT
Bax	Sense	TTGCTACAGGGTTTCATCCA
	Antisense	CTGAGACACTCGCTCAGCTT
Bcl-2	Sense	GAGTACCTGAACCGGCATCT
	Antisense	GAAATCAAACAGAGGTGCGCA
GAPDH	Sense	ACAGCAACAGGGTGGTGGAC
	Antisense	TTTGAGGGTGCAGCGAAGCTTT

NRF-1, nuclear respiratory factor 1; mtTFA, mitochondrial transcription factor; HIF-1 $\alpha$ , hypoxia inducible factor-1 $\alpha$ ; mtSSB, mitochondrial single stranded DNA binding protein; Bax, Bcl-associated X protein; Bcl-2, B-cell lymphoma-2.

Clara, CA, USA) was used to detect cellular energy, and XF Cell Mito Stress Tests (Seahorse Bioscience; Agilent Technologies, Inc.) were used to detect oxygen consumption rate. Briefly, the day before the assay, 1x10<sup>6</sup> cells were seeded in Seahorse 24-well XF Cell Culture Microplates, and the cells were then allowed to grow overnight in a cell culture incubator containing 5% CO<sub>2</sub> and 95% air at 37°C. The cells were then treated with 400  $\mu$ M CoCl<sub>2</sub> at 37°C for 6 h. Additionally, a Hydrate Sensor Cartridge was placed in a non-CO<sub>2</sub> incubator (37°C) overnight. The day of the assay, cells were washed with XF Glycolysis Stress Test Assay Medium, stock compounds [1  $\mu$ M oligomycin, 2  $\mu$ M carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) and 0.5  $\mu$ M rotenone/antimycin A] were added, and the cartridge ports were loaded. Following setting up the program on an XF Controller, the plate was placed in the instrument for testing. OCR was measured for about 3 min to detect basic OCR. Following baseline measurements, OCR was detected after injection of oligomycin, then FCCP was injected and the maximal OCR was measured. Non-mitochondrial oxygen consumption level was detected after the injection of rotenone/antimycin A.

**Statistical analysis.** Data were expressed as the mean  $\pm$  standard error. Statistical significance was assessed by two-tailed Student's t-tests or one-way analysis of variance with post hoc comparisons using the Student-Newman-Keuls test with GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

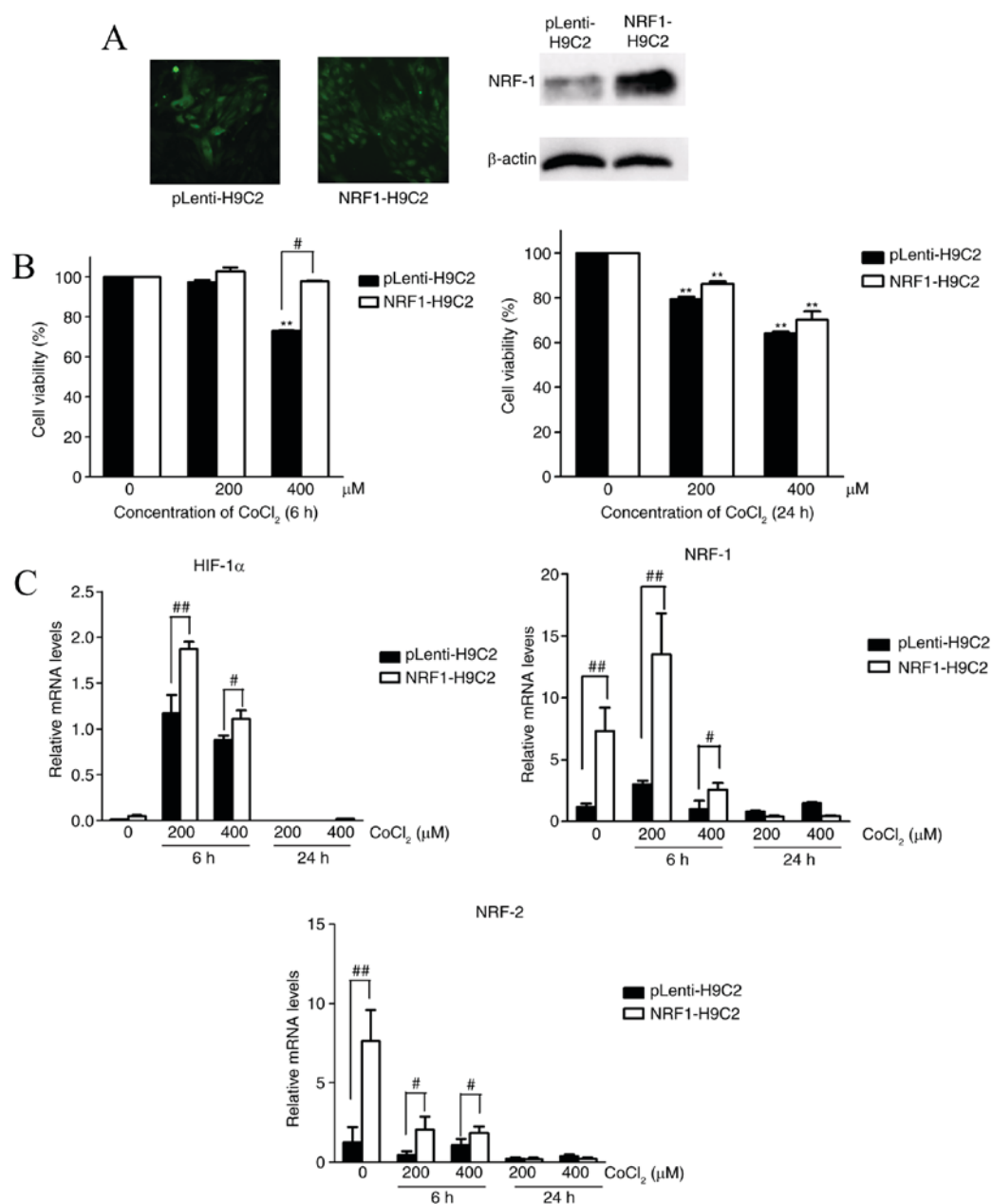


Figure 1. Effects of NRF-1 on the viability and mRNA expression levels of *HIF-1 $\alpha$* , *NRF-1* and *NRF-2* of H9C2 cells.  $\text{CoCl}_2$ -treated H9C2 cells were cultured in DMEM plus 10% FBS containing 200 or 400  $\mu$ M  $\text{CoCl}_2$  for 6 or 24 h. (A) GFP expression in lentiviral vector-transfected H9C2 cells, as observed by fluorescence microscopy (left); western blot analysis of NRF-1 expression levels in pLenti-H9C2 and NRF1-H9C2 cells (right). (B) CCK-8 assays were used to detect cell viability following treatment of pLenti-H9C2 and NRF1-H9C2 cells with 200 or 400  $\mu$ M  $\text{CoCl}_2$  for 6 or 24 h. (C) Reverse transcription-quantitative polymerase chain reaction was used to analyze *HIF-1 $\alpha$* , *NRF-1* and *NRF-2* mRNA expression levels in pLenti-H9C2 and NRF1-H9C2 cells. \*\* $P < 0.01$  vs. the 0  $\mu$ M  $\text{CoCl}_2$  group; \* $P < 0.05$ , ## $P < 0.01$  vs. pLenti-H9C2 cells.  $\text{CoCl}_2$ , cobalt chloride; HIF, hypoxia inducible factor; NRF, nuclear respiratory factor.

## Results

**Overexpression of NRF-1 alleviates the  $\text{CoCl}_2$ -induced decrease in cell viability.** To elucidate the role of NRF-1 in hypoxia caused by  $\text{CoCl}_2$  in H9C2 cells, H9C2 cells were transfected with NRF-1 overexpression lentiviral recombinant vector (NRF1-H9C2) or empty viral vector (pLenti-H9C2). Western blotting revealed that NRF-1 was upregulated in NRF1-H9C2 cells (Fig. 1A).

Subsequently, NRF1-H9C2 and pLenti-H9C2 cells were treated with 200 or 400  $\mu$ M  $\text{CoCl}_2$  for 6 or 24 h, and CCK-8 assays were used to detect cell viability. The results demonstrated that the  $\text{CoCl}_2$ -treated cells for 24 h had lower survival

ability than the untreated cells (Fig. 1B). NRF1-H9C2 cells treated with 400  $\mu$ M  $\text{CoCl}_2$  for 6 h demonstrated higher cell viability than pLenti-H9C2 cells. However, after 24 h treatment, there was no significant difference between NRF1-H9C2 and pLenti-H9C2 cells. RT-qPCR was performed to detect the mRNA expression of *NRF-1*, *NRF-2* and *HIF-1 $\alpha$* . As described above,  $\text{CoCl}_2$  can stabilize HIF-1 $\alpha$  (33,34). Furthermore, the data demonstrated that *HIF-1 $\alpha$*  was expressed at low levels in both groups under normal conditions, whereas treatment with 200 or 400  $\mu$ M  $\text{CoCl}_2$  for 6 h caused *HIF-1 $\alpha$*  mRNA levels to increase significantly in NRF1-H9C2 and pLenti-H9C2 cells, suggesting a response to hypoxia. However, it was also identified that the levels of *HIF-1 $\alpha$*  mRNA in NRF1-H9C2 cells



were higher compared with pLenti-H9C2 cells. One possible reason may be that NRF-1 possesses a direct regulation on *HIF-1a*, but NRF-1 had almost no elevation effect on *HIF-1a* in non- $\text{CoCl}_2$  treated cells, so another possible mechanism for the increased levels of *HIF-1a* in the experiments was that it was affected by some genes that were activated or inactivated by  $\text{CoCl}_2$ , and those genes were also influenced by NRF-1.  $\text{CoCl}_2$  can simulate hypoxia, which can also regulate the levels of HIF-1a. Theoretically, the levels of *HIF-1a* could be enhanced by  $\text{CoCl}_2$  in the experiments of the present study, but it is puzzling that 400  $\mu\text{M}$   $\text{CoCl}_2$  could not promote the levels of *HIF-1a* as did 200  $\mu\text{M}$   $\text{CoCl}_2$ , and that NRF-1 also dropped to a low levels in 400  $\mu\text{M}$   $\text{CoCl}_2$ -treated cells. Thus, the increase of *HIF-1a* levels caused by  $\text{CoCl}_2$  existed in certain conditions: A certain concentration of  $\text{CoCl}_2$  (200  $\mu\text{M}$ ) could cause stress effect of H9C2 cells in a short time (6 h), but when  $\text{CoCl}_2$  exceeded a certain level (400  $\mu\text{M}$  for 6 h, 200/400  $\mu\text{M}$  for 24 h), it would cause downstream effects on some genes like *NRF-1* and *HIF-1a*. Therefore NRF-1 and *HIF-1a* may have a special regulatory role in the presence of  $\text{CoCl}_2$ ; something which requires further study.

*Overexpression of NRF-1 can reduce apoptosis triggered by  $\text{CoCl}_2$ .* The decrease in H9C2 cell viability induced by  $\text{CoCl}_2$  may be due to apoptosis. Therefore, the nuclear morphology of  $\text{CoCl}_2$ -treated cells using Hoechst 33342 staining was first examined. As shown in Fig. 2A, round and weakly stained cells were observed prior to treatment. However,  $\text{CoCl}_2$ -treated cells demonstrated clear pyknosis and strong blue staining. The number of apoptotic nuclei was significantly lower in NRF1-H9C2 cells compared with that in pLenti-H9C2 cells.

Subsequently, flow cytometry was performed to analyze apoptosis in NRF1-H9C2 and pLenti-H9C2 cells. The results demonstrated that the apoptosis rates of both types of  $\text{CoCl}_2$ -treated cells were significantly increased, although NRF1-H9C2 cells exhibited a relatively low rate of apoptosis (Fig. 2B). The expression of the Bcl-2 and Bax were also detected (Fig. 2C and D). Notably, in NRF1-H9C2 cells, the expression of the Bcl-2, which encodes an anti-apoptotic molecule, maintained a higher level compared with that in pLenti-H9C2 cells. The expression of Bax, which encodes a pro-apoptotic molecule, was increased in  $\text{CoCl}_2$ -treated cells; however, there were no obvious differences between NRF1-H9C2 and pLenti-H9C2 cells, which might indicate that NRF-1 has no inhibitory effect on Bax. The levels of PARP, which is the cleavage substrate of caspase, were higher in  $\text{CoCl}_2$ -treated pLenti-H9C2 cells compared with that in NRF1-H9C2 cells. Based on these findings, the decrease in H9C2 cell viability may be partially due to the induction of apoptosis by  $\text{CoCl}_2$ , and the presence of NRF-1 may have an inhibitory effect on apoptosis.

*Effects of NRF-1 on MMP in  $\text{CoCl}_2$ -treated H9C2 cells.* Multiple studies have demonstrated that MMP is an important index of apoptosis (45-48), MMP plays an important role in mitochondrial function and decline of MMP caused by stress can lead to mitochondrial dysfunction. In Fig. 3A, the red curve represented the untreated cells and the blue curve represented the  $\text{CoCl}_2$ -treated cells, which suggested the reduction of

MMP level. In normal conditions, MMP in both groups maintained in high levels. However, changes in MMP level in both groups were observed following treatment with 400  $\mu\text{M}$   $\text{CoCl}_2$  for 6 h, which exhibited the decline of MMP level, revealing the damaging effects of  $\text{CoCl}_2$  on mitochondrial function. The results of the present study also demonstrated that MMP levels in pLenti-H9C2 cells was lower compared with NRF1-H9C2 cells. A previous study identified that apoptosis was accompanied by the decline of MMP (45), data from the present study demonstrated that the overexpression of NRF-1 could alleviate the decrease of MMP, suggesting a protective effect of NRF-1 on mitochondria. In addition, *mtTFA* and *mtSSB* mRNA levels were significantly altered following treatment with  $\text{CoCl}_2$ , with the mRNA levels remaining higher in NRF1-H9C2 cells than in pLenti-H9C2 cells. These data indicated that overexpression of NRF-1 could alleviate the decrease in MMP, potentially by affecting the expression levels of mitochondria-associated genes, such as *mtTFA* and *mtSSB* (Fig. 3B).

*Overexpression of NRF-1 increases the oxygen consumption in  $\text{CoCl}_2$ -treated H9C2 cells.* OCRs were used as an index of mitochondrial respiratory capacity (49,50). Under normal conditions, OCRs were similar in NRF1-H9C2 and pLenti-H9C2 cells, suggesting that there were no differences in mitochondrial respiratory capacity between the two groups (Fig. 4A and B) under normal conditions. Following treatment of cells with 400  $\mu\text{M}$   $\text{CoCl}_2$  for 6 h, the basic and maximum OCRs were decreased in both NRF1-H9C2 and pLenti-H9C2 cells, although the OCR in NRF1-H9C2 cells was relatively higher than that in pLenti-H9C2 cells. The above results indicated that NRF-1 may enhance the mitochondrial respiratory capacity to resist  $\text{CoCl}_2$ -dependent impairment in H9C2 cells.

## Discussion

Hypoxia is a common cause of pathological damage to organs, including the heart, where brief and rapid hypoxia can cause irreversible damage. The heart is a unique organ that pumps blood into various tissues, and compared with other parts of the body, hypoxia in the heart results in serious consequences (7,8). Cardiomyocytes are the structural and functional units of the heart (51,52). Because adult cardiomyocytes lose their ability to proliferate (52-54), H9C2 cells, a proliferating cardiomyocyte cell line, has often used to observe the effect of hypoxia on cardiomyocytes. Many types of hypoxia can be attributed to the loss of oxygen caused by inadequate tissue perfusion or a drop in systemic oxygen partial pressure in the arterial blood (55). As described above,  $\text{CoCl}_2$  is a well-known hypoxia-mimetic chemical reagent that is widely used in many hypoxia studies (56-59). In the present study, H9C2 cardiomyocytes were exposed to two different concentrations of  $\text{CoCl}_2$ , which resulted in a decrease in cell viability consistent with a previous study in which  $\text{CoCl}_2$  was reported to behave as a cytotoxic agent (60,61). The present data also demonstrated the protective effects of NRF-1 on  $\text{CoCl}_2$ -treated H9C2 cells, an effect that had not been previously reported. Although multiple studies have reported that NRF-1 has protective effects on cells under stress conditions (62-64), these findings led us to explore the role of NRF-1, and particularly the protective mechanisms of NRF-1 in  $\text{CoCl}_2$ -treated cells.

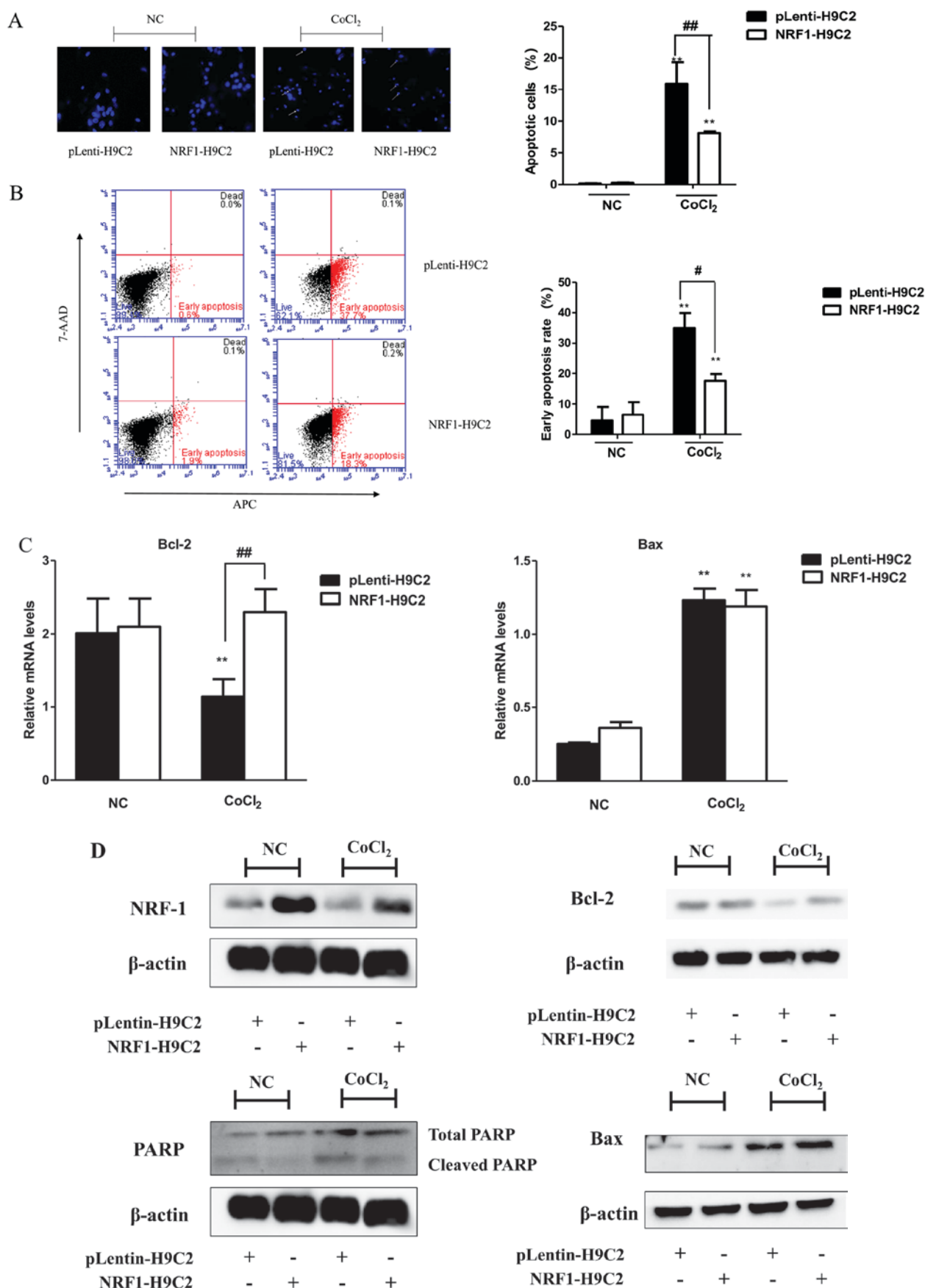


Figure 2. Effects of CoCl<sub>2</sub> on apoptosis. (A) Changes in nuclear morphology, representative of apoptosis, in cells induced by CoCl<sub>2</sub> (400 μM) for 6 h were observed by Hoechst 33342 staining in pLenti-H9C2 and NRF1-H9C2 cells. Cells with intense blue staining in nuclei were counted. Magnification, x400. (B) Flow cytometry was used to detect apoptosis following staining with Annexin V-APC and 7-ADD. (C) mRNA expression of *Bcl-2* and *Bax* was analyzed by reverse transcription-quantitative polymerase chain reaction. (D) Protein expression levels of NRF-1, Bcl-2, Bax and PARP were analyzed by western blot. \*\*P<0.01 vs. NC; #P<0.05, ##P<0.01 vs. pLenti-H9C2 cells. NC, negative control (CoCl<sub>2</sub> untreated cells); CoCl<sub>2</sub>, cobalt chloride; NRF, nuclear respiratory factor; 7-AAD, 7-aminoactinomycin D; APC, allophycocyanin; Bcl-2, B-cell lymphoma-2; Bax, Bcl-associated X protein; PARP, poly(ADP-ribose) polymerase 1.

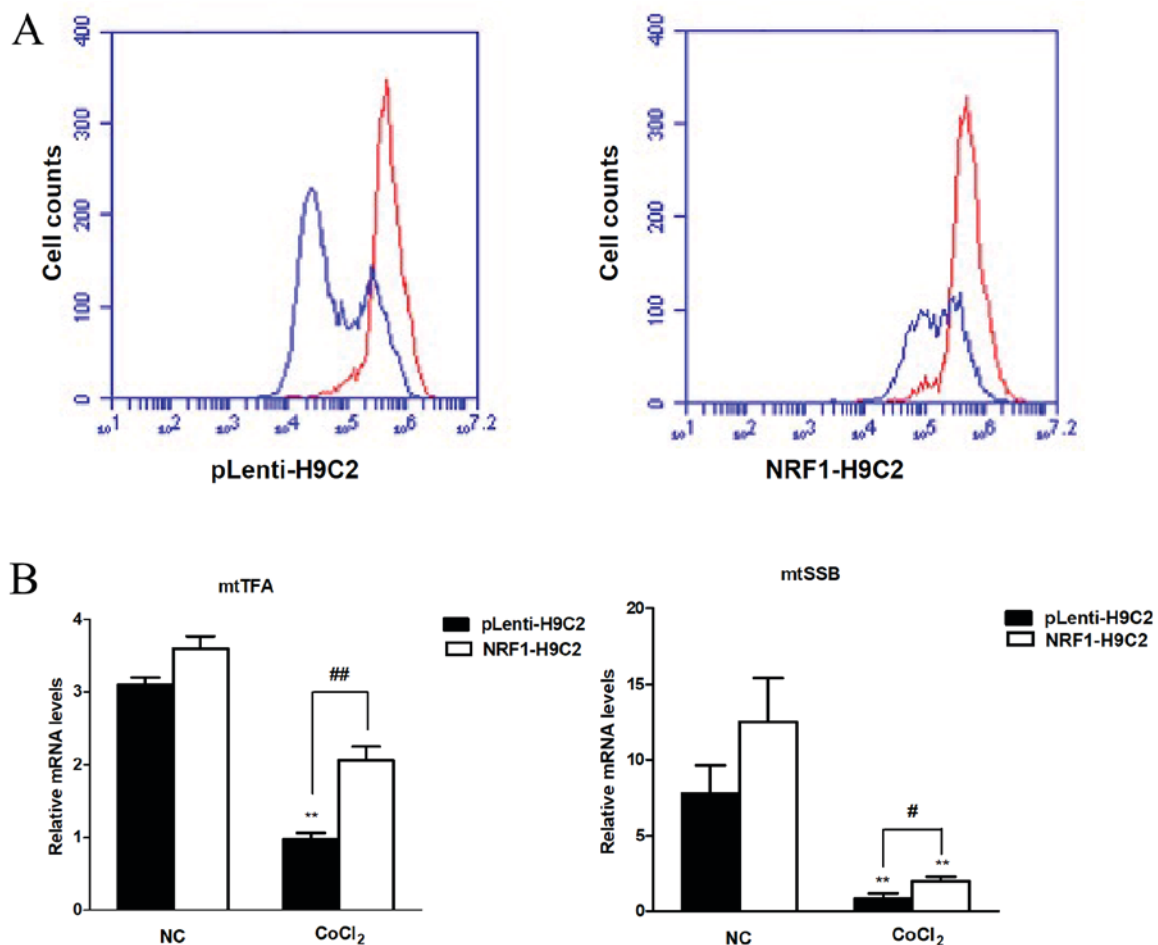


Figure 3. Effects of CoCl<sub>2</sub> on mitochondrial membrane potential and the mRNA expression of *mtTFA* and *mtSSB* in H9C2 cells. (A) Changes in mitochondrial membrane potential in pLenti-H9C2 and NRF1-H9C2. The cells were treated with 400  $\mu$ M CoCl<sub>2</sub> for 6 h. The red curve represented the untreated cells, the blue curve represented the CoCl<sub>2</sub>-treated cells. (B) Reverse transcription-quantitative polymerase chain reaction was used to analyze *mtTFA* and *mtSSB* mRNA expression levels in pLenti-H9C2 and NRF1-H9C2 cells. \*\* $P < 0.01$  vs. NC group (CoCl<sub>2</sub> untreated cells); # $P < 0.05$ , ## $P < 0.01$  vs. pLenti-H9C2 cells. NRF, nuclear respiratory factor; CoCl<sub>2</sub>, cobalt chloride; mtTFA, mitochondrial transcription factor; mtSSB, mitochondrial single stranded DNA binding protein; NC, negative control.

NRF-1 is involved in the integration of nuclear and mitochondrial genes (20,22); however, few studies have evaluated the protective effects of NRF-1 on CoCl<sub>2</sub>-induced cell damage (65). NRF-2, together with NRF-1, serves a key role in the integration of nuclear and mitochondrial genomes by directly or indirectly regulating the expression of nuclear-encoded genes (66-68). Various studies have focused on NRF-2, which has been described as a 'multi-organ protector' and can protect against many diseases in a number of organs (69), including chronic obstructive pulmonary disease, nephrotoxicity from ischemia-reperfusion injury and cisplatin and cardiomyopathy induced by smoking (70). Furthermore, inhibition of NRF-2 can render cells susceptible to apoptosis (71,72) and has been shown to weaken endoplasmic reticulum stress-induced apoptosis in some experiments (73,74). Additionally, NRF-2 is a crucial regulator of the response to cobalt and can be activated in response to CoCl<sub>2</sub>-mediated oxidative stress (75). In the present study, both NRF-2 and NRF-1 increased in CoCl<sub>2</sub>-treated H9C2 cells, a previous study demonstrated that there are four antioxidant-responsive elements in the promoter region of *NRF-1* that can be regulated by NRF-2, leading to an increase of NRF-1 protein levels (76); thus, whether the results

of the present study indicated that there exists an interaction between NRF-1 and NRF-2, or whether their increase does not depend on each other in CoCl<sub>2</sub>-treated cells but is a mutual independence, requires more precise data to elucidate. The findings of the present study also indicated that the reduced proliferation of CoCl<sub>2</sub>-treated H9C2 cells was partially due to the occurrence of apoptosis, and overexpression of NRF-1 alleviated CoCl<sub>2</sub>-induced apoptosis; this pro-survival effect was consistent with other studies (63,76), suggesting that NRF-1 has an anti-apoptotic role, similar to NRF-2 (70).

Bcl-2 and Bax are two important apoptotic factors that are part of the Bcl-2 family, although they are classified into different groups based on their functions and structures: Bcl-2 is an anti-apoptotic molecule, whereas Bax is a pro-apoptotic molecule, an entry point for the intrinsic apoptotic signaling pathway (77), and can be activated by various stimuli, ultimately leading to cell death (77-80). Bax activates caspase-9 (81), regulates the unfolded protein response by interacting with inositol requiring 1- $\alpha$  (82) and also modulates mitochondrial function (83). Bax and Bcl-2 are highly homologous and can form heterodimers (84). A number of studies have suggested that Bcl-2 is an anti-apoptotic protein (85-88).

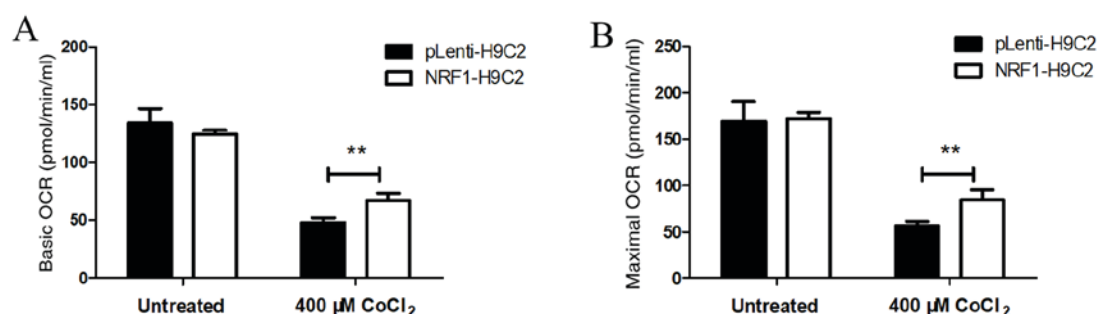


Figure 4. Detection of mitochondrial OCRs. (A) The basic and maximum OCR in untreated cells was detected. (B) The basic and maximum OCR in cells treated with 400  $\mu$ M CoCl<sub>2</sub> for 6 h was detected. \*\*P<0.01 vs. pLenti-H9C2 cells. FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; CoCl<sub>2</sub>, cobalt chloride; NRF, nuclear respiratory factor.

Therefore, the opposing roles of Bcl-2 and Bax are important in studies of apoptosis. Levels of *Bax* were increased in CoCl<sub>2</sub>-treated cells, and *Bcl-2* levels reduced in CoCl<sub>2</sub>-treated pLenti-H9C2. Notably, no differences were observed in Bax expression between pLenti-H9C2 and NRF1-H9C2 cells, suggesting that the inhibitory effects of NRF-1 on apoptosis may be associated with the transcriptional activation of Bcl-2. Furthermore, expression levels of the cleaved PARP were lower in NRF1-H9C2 cells compared with the pLenti vector control following treatment with CoCl<sub>2</sub>. These protective effects indicated that NRF-1 may mediate resistance to apoptosis. Additionally, when the incubation time was increased to 24 h, the cytoprotective effects of NRF-1 were not observed, possibly because the notable decrease in *NRF-1* removed the defense against CoCl<sub>2</sub> damage.

The main mechanisms of apoptosis are the mitochondrial pathway and the death receptor pathway. Stress-induced mitochondrial dysfunction can severely affect cell survival and result in apoptotic cell death (89-93). NRF-1 was originally identified in a study of the promoter activity of cytochrome *c* (94,95), an electron transport vehicle that can shuttle between different complexes; this feature has become an important target for the study of transcriptional regulation of various mitochondria-associated genes (96). Further in-depth studies have revealed that there are NRF-1 recognition sites in the promoter regions of several nuclear genes required for the expression of respiratory genes (97-101). Thus, the anti-apoptotic and pro-survival effects of NRF-1 on CoCl<sub>2</sub>-treated H9C2 cells may be associated with the maintenance of mitochondrial function. Previous studies have demonstrated that MMP has a critical role in the maintenance of mitochondrial function (102,103). Disruption of MMP can cause cytochrome *c* to enter the cytosol, inducing apoptosis (104-106). The present findings indicated that overexpression of NRF-1 reduced the depolarization of MMP caused by 400  $\mu$ M CoCl<sub>2</sub>. In addition, the present results demonstrated that NRF-1 may enhance the expression of the *mtTFA* gene, consistent with previous studies (99). mtTFA is important for the initiation and transcription of mitochondria-encoded genes (107-109). These data suggest that in response to injury caused by CoCl<sub>2</sub> and to maintain adequate mitochondrial functionality, NRF-1 can increase the expression of mitochondria-associated genes, mtTFA and mtSSB, thereby regulating mtDNA transcription and replication (110-112), and protecting the cell from stress.

MMP can effectively promote the synthesis of ATP by generating electrochemical gradients. It was speculated that disruption of MMP may inhibit the cellular mitochondrial respiratory capacity. In the present study, mitochondrial respiration was investigated by detecting the OCR of CoCl<sub>2</sub>-treated NRF1-H9C2 and pLenti-H9C2 cells. Mitochondrial respiratory potential has been measured as an index of oxidative stress-mediated mitochondrial dysfunction in previous studies (49,113). In the present study, there was a significant decrease in mitochondrial respiration in CoCl<sub>2</sub>-treated cells, and overexpression of NRF-1 subsequently enhanced mitochondrial respiration. Thus, these findings revealed that NRF-1 may be involved in the regulation of mitochondrial function.

In summary, NRF-1 acted as an important anti-apoptotic molecule in CoCl<sub>2</sub>-treated H9C2 cells, potentially by reducing the decrease in MMP and increasing mitochondrial respiration to strengthen cellular adaptability in the context of CoCl<sub>2</sub>-induced hypoxia.

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### Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

### Authors' contributions

NN, WZ, ZHL and RS contributed to writing the manuscript and revision. RS and WZ designed and revised the experiments. NN and ZHL wrote the manuscript and performed cell experiments. NN, HLS and SMX performed detection of energy metabolism. ZHL and MXZ prepared the images. MXZ performed the packaging of the virus. JHY performed flow cytometry assay.



## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that there are no competing interests.

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