

## Original Paper

# FGF-2 Transcriptionally Down-Regulates the Expression of BNIP3L *via* PI3K/Akt/FoxO3a Signaling and Inhibits Necrosis and Mitochondrial Dysfunction Induced by High Concentrations of Hydrogen Peroxide in H9c2 Cells

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**Key Words**

H<sub>2</sub>O<sub>2</sub> • Necrosis • FGF-2 • PI3K/AKT/FoxO3a • BNIP3L • Mitochondrial dysfunction

**Abstract**

**Background/Aims:** Cardiovascular disease is a growing major global public health problem. Necrosis is one of the main forms of cardiomyocyte death in heart disease. Oxidative stress is regarded as one of the key regulators of cardiac necrosis, which eventually leads to cardiovascular disease. Many pharmacological and *in vitro* studies have suggested that FGF-2 can act directly on cardiomyocytes to maintain the integrity and function of the myocardium and prevent damage during oxidative stress. However, the mechanisms by which FGF-2 rescues the myocardium from oxidative stress damage in cardiovascular disease remain unclear. The present study explored the protective effects of FGF-2 in the H<sub>2</sub>O<sub>2</sub>-induced necrosis of H9C2 cardiomyocytes as well as the possible signaling pathways involved. **Methods:** Necrosis of H9c2 cardiomyocytes was induced by H<sub>2</sub>O<sub>2</sub> and assessed using a Cell Counting Kit-8 (CCK8) assay and flow cytometry analysis. The cells were pretreated with the PI3K/Akt inhibitor Wortmannin to investigate the possible involvement of the PI3K/Akt pathway in the protection by FGF-2. The levels of Akt, p-Akt, FoxO3a, p-FoxO3a, and BNIP3L were detected by Western blot. Chromatin immuno-precipitation (ChIP) analysis was used to test whether FoxO3a binds directly to the BNIP3L promoter region. A luciferase assay was used to study the effects of FoxO3a on BNIP3L gene promoter activity. Mitochondrial ΔΨM was

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quantified using tetramethylrhodamine methyl ester perchlorate (TMRM). The mitochondrial oxygen consumption rate (OCR) was assessed with a Seahorse XF24 Analyzer. **Results:** Treatment with H<sub>2</sub>O<sub>2</sub> decreased the phosphorylation of Akt and FoxO3a, and it induced the nuclear localization of FoxO3a and the necrosis of H9c2 cells. These effects of H<sub>2</sub>O<sub>2</sub> were abrogated by pretreatment with FGF-2. Furthermore, the protective effects of FGF-2 were abolished by the PI3K/Akt inhibitor Wortmannin. ChIP analyses indicated that FoxO3a binds directly to the BNIP3L promoter region. Using a luciferase assay, we further observed that FoxO3a increased BNIP3L gene promoter activity. As expected, overexpression of BNIP3L in H9C2 cardiomyoblast cells reduced the cardioprotection of FGF-2 in H<sub>2</sub>O<sub>2</sub>-induced necrosis and mitochondrial dysfunction. **Conclusions:** The present data suggest that FGF-2 protects against H<sub>2</sub>O<sub>2</sub>-induced necrosis of H9C2 cardiomyocytes via the activation of the PI3K/Akt/FoxO3a pathway. Moreover, the present results demonstrate that FoxO3a is an important transcription factor that acts by binding to the promoter and promoting the transcription of BNIP3L, and it contributes to the necrosis and mitochondrial dysfunction induced by H<sub>2</sub>O<sub>2</sub> in H9c2 cardiomyoblast cells.

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## Introduction

Myocardial ischemia/reperfusion injury is one of the most common causes of death worldwide [1-6]. Numerous studies have demonstrated that cell death is an important component in the pathogenesis of various cardiac diseases, including heart failure, myocardial infarction (MI) and ischemia/reperfusion (I/R) [7-10]. Myocytes injured during heart disease include both apoptotic and necrotic cells. Studies have shown that necrosis is more prominent in failing hearts and I/R hearts, which indicated that necrosis plays an important role in the pathological process of cardiac disease [11-17]. Oxidative stress is defined as an excess production of reactive oxygen species (ROS) relative to the endogenous antioxidant reserve to counteract the effects of ROS [18-22]. Several studies have indicated that ROS induce the necrosis of cardiac myocytes and play a pivotal role in cardiac pathophysiology responsible for the development and progression of ischemic heart disease [17, 23, 24]. Nevertheless, the underlying mechanisms of cardiomyocyte necrotic death are not fully understood. Therefore, clarification of the mechanism of myocardial cell necrosis after oxidative stress would be helpful and might provide new targets for the treatment of ischemic heart disease.

Fibroblast growth factor 2 (FGF-2), which is also known as basic FGF, is a member of the fibroblast growth factor family [25]. FGF-2 is expressed in all developmental stages of the heart and confers cardioprotection against myocardial ischemia [26]. Several studies suggest that the protective effects of FGF-2 are mediated by PKC and MAPK activation [27]. Moreover, the PI3K/AKT pathway, which is independent of the PKC, PKA, and MAPK signaling pathways [28, 29], is activated by FGF-2 and prevents ROS-induced apoptosis in H9c2 cells through the Forkhead box O3 (FoxO3a) transcription factor [30]. The FoxO3a transcription factor is one of the most important downstream targets of PI3K/Akt signaling and a crucial regulator of apoptosis [18, 31-33]. Sodium tanshinone IIA sulfonate [34] and bromelain [35] were reported to protect rat hearts from ischemia-reperfusion injury through activation of the PI3K/Akt/FoxO3a pathway. However, the roles of FGF-2 and the PI3K/AKT/FoxO3a signaling pathway in ROS-induced necrosis are not clear. Taking all these factors into consideration, the present study aimed to identify the function and mechanisms of FGF-2 in ROS-induced necrosis in H9c2 cells.

## Materials and Methods

### Cell culture

H9c2 cardiomyoblasts were obtained from the American Type Culture Collection (Manassas, VA). H9c2 cells were cultured in Dulbecco's modified Eagle's medium in 100-mm culture dishes and supplemented

with 10% fetal bovine serum, 100 U/mL penicillin, 2 mM glutamine, 100 µg/mL streptomycin, and 1 mM HEPES buffer. The cultures were incubated at 37°C in humidified air with 5% CO<sub>2</sub>. The medium was replaced every other day. To determine the percentage of necrotic cells, H9c2 cardiac myocytes were pretreated with recombinant rat FGF-2 protein (Sigma-Aldrich, MO, USA) for 30 min before a 6 h treatment with H<sub>2</sub>O<sub>2</sub>. In some experiments, H9c2 cells were pretreated with the phosphatidylinositol-3-kinase (PI3K) inhibitor Wortmannin (1 µM, Thermo Fisher, CA, USA) before FGF-2 stimulation. BNIP3L plasmids were procured from OriGene (Level Biotechnology Inc., Taipei, Taiwan), and H9c2 cells were transfected using PureFection reagents (System Biosciences, CA, USA) for 24 h.

### *Cell viability assay*

At the end of the indicated time, H9c2 cells were treated with CCK8 (10 µl/well, Sigma, USA) for an additional 2 h. Finally, we recorded the absorbance at 450 nm using a microplate absorbance reader (Tecan, Safire II, Switzerland).

### *Cell necrosis Assays*

Cell death was measured with the Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit (Thermo Fisher, CA, USA), and testing was performed on a FACSCalibur device (Becton Dickinson, NJ, USA). All PI positive cells (PI+) were considered necrotic cells, whereas Annexin V positive/PI negative cells (Annexin V+/PI-) were considered apoptotic cells.

### *Western Blot*

Cells were lysed for 30 min on ice in Radio-Immunoprecipitation Assay (RIPA) lysis buffer (Solarbio, Beijing, China) containing 0.1 mM PMSF and a protease inhibitor (Roche). The samples were subjected to 12 % SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed using primary antibodies, including anti-phospho-AKT (Abcam, Cambridge, UK), anti-AKT (Abcam), anti-phospho-FoxO3a (Abcam), anti-FoxO3a (Abcam), anti-histone 3 (Earthox LLC., San Francisco, CA, USA), anti-BNIP3L (Abcam) and anti-β-actin (Santa Cruz Biotechnology). After four washes with PBS-Tween 20, horseradish peroxidase-conjugated secondary antibodies were added. The signals were detected with Pierce® ECL Western blotting substrate (Pierce, Rockford, IL, USA) according to the directions of the manufacturer and using X-ray films (Kodak, Rochester, NY, USA).

### *Chromatin immunoprecipitation (ChIP) assay*

A ChIP assay was performed as described previously. Briefly, cells were crosslinked with formaldehyde, and chromatin was fragmented by sonication. Chromatin was immunoprecipitated with anti-FoxO3a (Santa Cruz) or control IgG, and purified co-precipitated DNA was quantified by PCR with Ex Taq™ Polymerase (TaKaRa, Otsu, Japan). The PCR products were then analyzed using agarose gel electrophoresis and EtBr staining for visualization. The primers used to amplify the DNA fragments were forward 5'-TCCCATCATTAGCTGTCCCCAAA-3' and reverse 5'-CGGTGCGAGATGCCTTTTGAA-3'.

### *Luciferase assay*

The BNIP3L-luciferase constructs were created by inserting ~3.2 kb and ~2.9 kb fragment encompassing the predicted binding site into the pGL4-BASIC-luciferase plasmid (Promega, Tokyo, Japan). The primers used to amplify the DNA fragments were BNIP3L\_P1 (forward 5'-TTCCCATCATTAGCTGTCCCC-3' and reverse 5'-GTTCTGAGCACACCTCCTAC-3') and BNIP3L\_P2 (forward 5'-AGGAACGCTTATTTTCTGACCA-3' and reverse 5'-ATGTTCTGAGCACACCTCC-3'). A commercial plasmid containing a CMV-driven Renilla reporter system was used as an internal control (Promega). H9c2 cells were plated in 6-well plates at 50–70% confluence and were co-transfected with the pCMV-BNIP3L construct or with an equimolar amount of the empty pCMV vector and the pGL4-BNIP3L-P1 or pGL4-BNIP3L-P2 construct utilizing PureFection™ reagents (System Biosciences). The media was changed 2 h prior to transfection. After the media was changed, the cells were incubated in 10% DMEM for 24 h. The luciferase assays were performed using the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega). Briefly, 100 µl of luciferase substrate was added to 20 µl of lysate, and luciferase activity was measured using an LB940 Multilabel Reader (Berthold Technologies, Bad Wildbad, Germany). Each luciferase assay was performed in triplicate.

### Mitochondrial $\Delta\Psi$ assay

Mitochondrial  $\Delta\Psi$  was assessed by epifluorescence microscopy after incubating cells with 50 nM tetra-methylrhodamine methyl ester perchlorate (TMRM) (Molecular Probes) [36].

### Mitochondrial Respiration assay

Mitochondrial OCR was assessed with a Seahorse XF24 Analyzer. In brief, H9c2 cardiomyocytes (8000-10000 cells per well) were cultured in 24-well plates and culture medium was changed 1 hour before the assay to unbuffered DMEM medium (pH 7.4) supplemented with 1 mM pyruvate (Thermo Fisher), 10 mM D-glucose. Oligomycin (1  $\mu$ M), FCCP (2-[2-[4-(trifluoromethoxy)phenyl]hydrazinylidene]-propanedinitrile) (1  $\mu$ M), and rotenone (1  $\mu$ M) combined with antimycin (1  $\mu$ M) were injected sequentially through ports in the Seahorse Flux Park cartridges as previously reported [37]. First, the basal oxygen consumption rate (basal respiration) was measured. Oligomycin inhibited ATP synthase activity, which led to the development of a proton gradient that inhibited electron flux and revealed the state of the coupling efficiency. FCCP uncoupled the respiratory chain and revealed the maximal capacity for reducing oxygen. The spare respiratory capacity was calculated by subtracting the basal respiration from the maximal respiration. Finally, rotenone combined with antimycin A was injected to inhibit the flux of electrons through complexes I and III; the remaining oxygen consumption rate was primarily due to non-mitochondrial respiration. After OCR measurement, the cells were fixed and stained with the Hoechst 33258 nuclear dye. The plates were scanned to quantify cell numbers using a Cellomics ArrayScan VTI HCS Reader (Thermo Scientific). OCR was normalized to cell number per respective well.

### Statistical Analysis

Multiple comparisons between groups were tested using a one-way ANOVA. Bonferroni post hoc tests were used to determine differences among the groups. The unpaired two-tailed Student t-test was used to compare differences between the means of two groups. Differences were considered to be statistically significant at a level of  $p < 0.05$ .

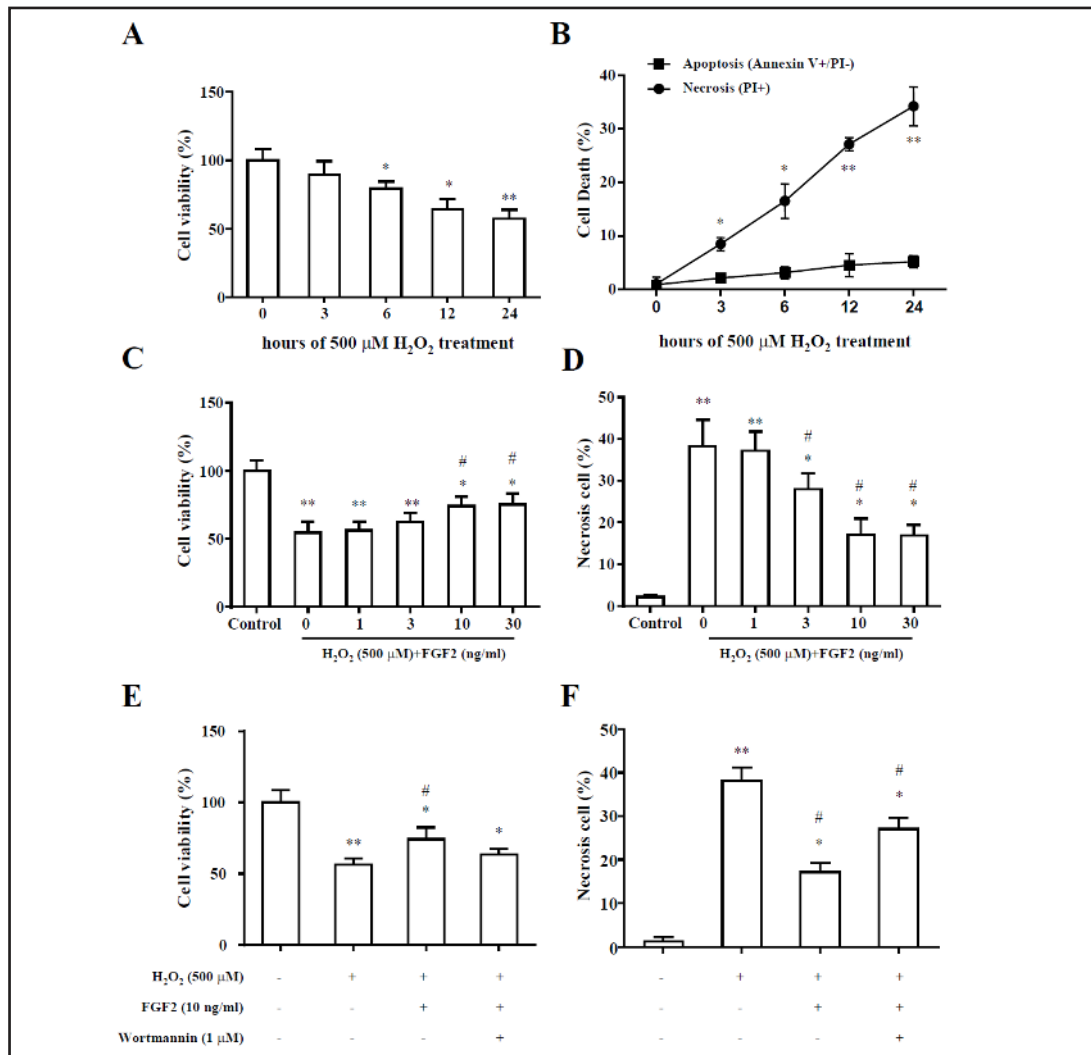
## Results

### *FGF-2 protects H9c2 cardiomyocytes against H<sub>2</sub>O<sub>2</sub>-induced necrosis involving the PI3K/Akt pathway*

H<sub>2</sub>O<sub>2</sub> is known to be an important factor for inducing both apoptosis and necrosis. Its role depends on the exact concentration that is applied and the cell type tested. We tested the effects of H<sub>2</sub>O<sub>2</sub> on the death of the heart cell line H9C2 at different times. After incubating with H<sub>2</sub>O<sub>2</sub> (500  $\mu$ M) for 0, 3, 6, 12, and 24 h, we observed a time-dependent decrease in cell viability (Fig. 1A) and an increase in necrosis (Fig. 1B). To investigate the effects of FGF-2 in H<sub>2</sub>O<sub>2</sub>-induced necrosis, Fig. 1C shows that cell viability fell to 54 % after exposure to H<sub>2</sub>O<sub>2</sub> (500  $\mu$ M) for 24 h. However, pretreatment with 10 or 30 ng/ml FGF-2 for 30 min prior to exposure to H<sub>2</sub>O<sub>2</sub> significantly ameliorated the H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity, as shown by the increase in cell viability. The percentage of cells stained by PI revealed that 38 % of cells experienced necrotic death after 24 h of exposure to H<sub>2</sub>O<sub>2</sub>. FGF-2 treatment led to a significant 17 % decrease in cardiomyocyte necrosis (Fig. 1E) compared to the H<sub>2</sub>O<sub>2</sub>-induced control group. Figure 1E shows that cell viability was reduced to 56 % by H<sub>2</sub>O<sub>2</sub>, whereas a 10 ng/ml FGF-2 pretreatment significantly increased cell viability to 74 %. Compared to the FGF-2 group, the cell viability of the FGF-2 + H<sub>2</sub>O<sub>2</sub> + Wortmannin group decreased to 64 %. Necrosis was significantly and consistently increased to 27 % (Fig. 1F) by co-treatment with FGF-2 and Wortmannin compared to FGF-2 treatment alone. These data strongly suggested that FGF-2 has a protective role in H<sub>2</sub>O<sub>2</sub>-induced necrotic cell death of H9c2 cells, which may involve the PI3K/Akt pathway.

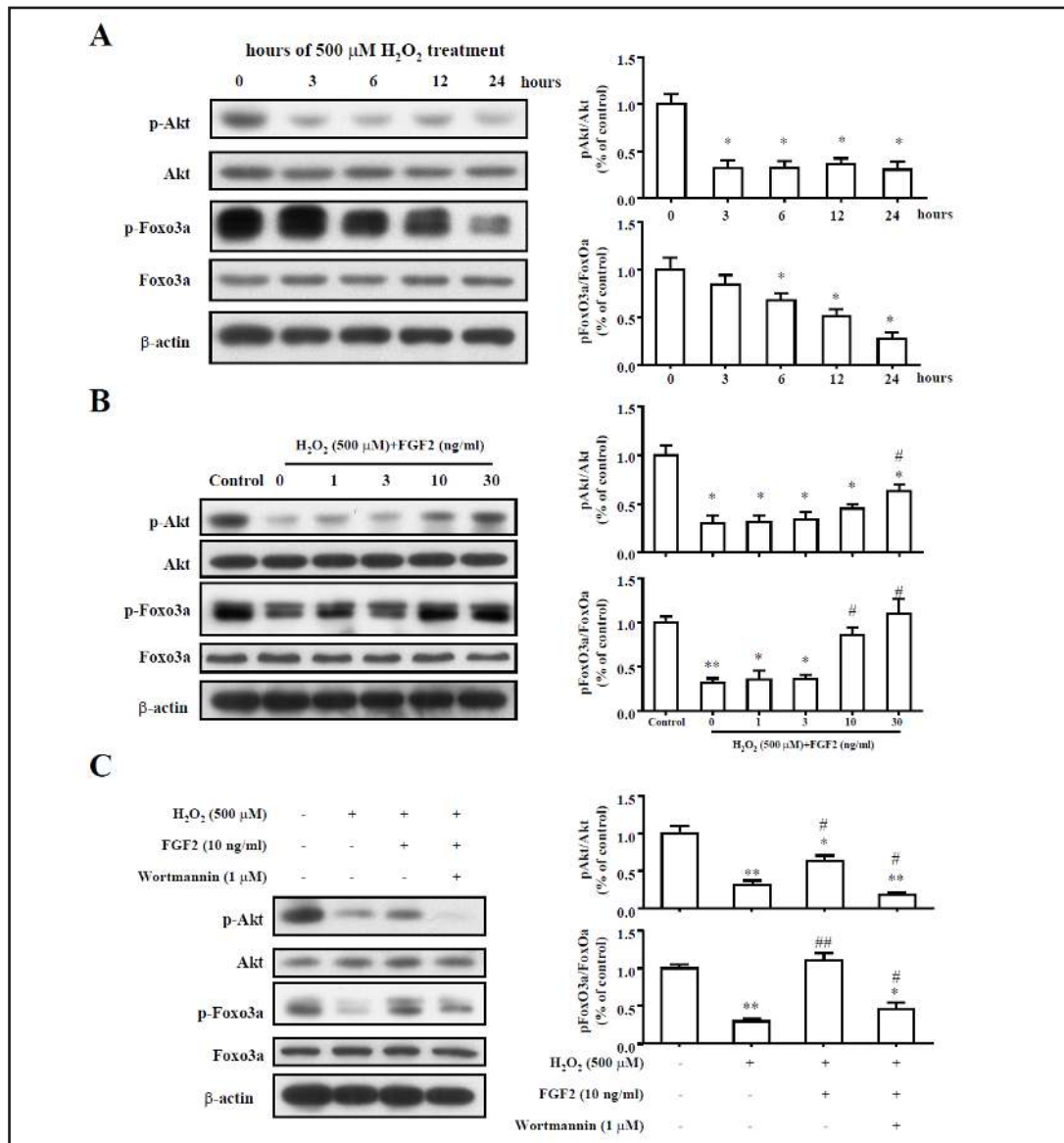
### *Phosphorylation of Akt and FoxO3a proteins is involved in the cardioprotection of FGF-2 against H<sub>2</sub>O<sub>2</sub>-induced necrosis in H9c2 cells*

To investigate the role of PI3K/Akt/FoxO3a in mediating the effects of H<sub>2</sub>O<sub>2</sub> in the necrosis of H9c2 cells, the phosphorylation of Akt and FoxO3a in H9c2 cells was studied



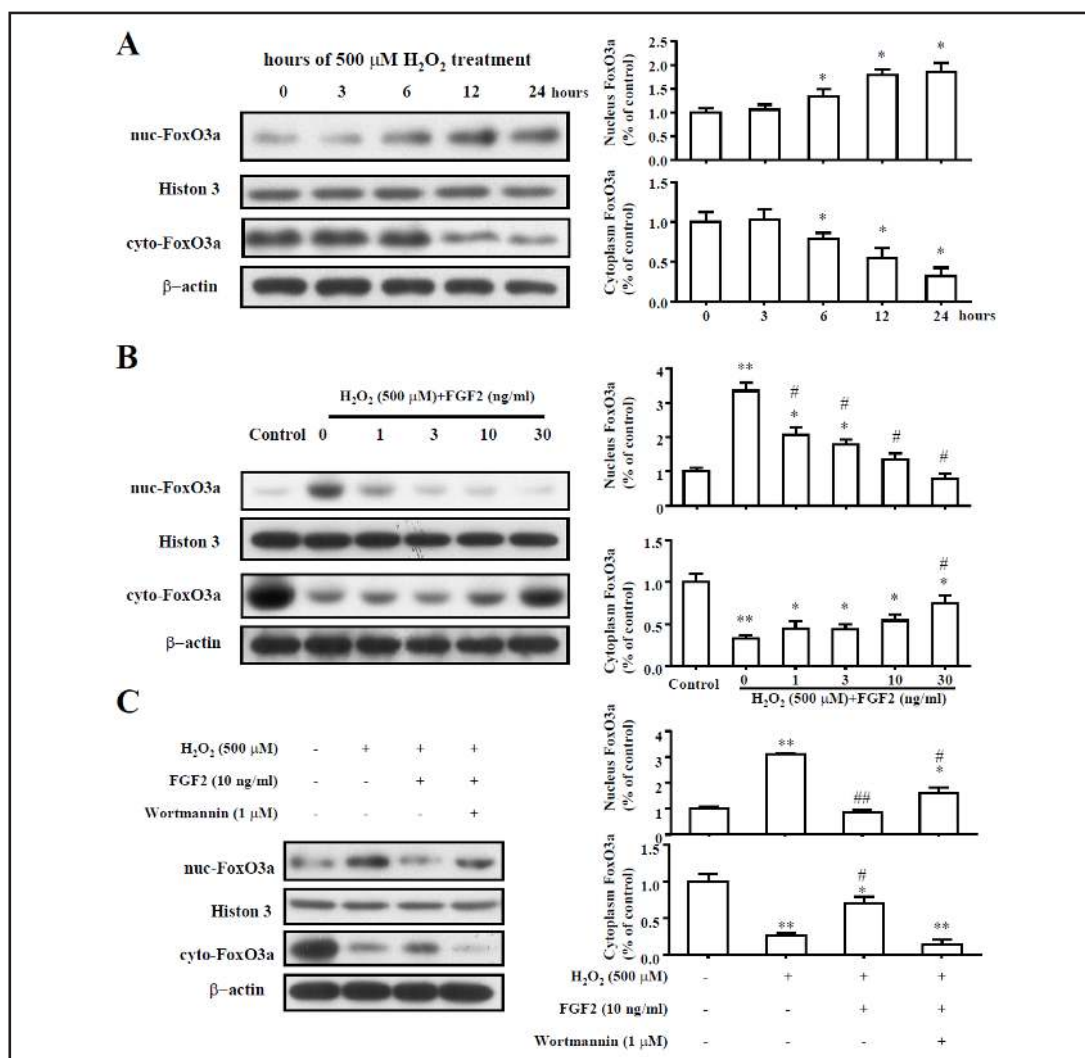
**Fig. 1.** Protective effects of FGF-2 against H<sub>2</sub>O<sub>2</sub>-induced necrosis in H9c2 cardiomyocytes involved the PI3K/AKT pathway. (A) Cell viability was analyzed by a CCK8 assay in H9c2 cells treated with H<sub>2</sub>O<sub>2</sub> (500 μM) for 0, 3, 6, 12, or 24 hours; Data are mean ± SD (n = 5). \*p < 0.05, \*\*p < 0.01 versus the control group. (B) Apoptotic and necrotic cells were analyzed by flow cytometry using the Annexin V/PI assay in H9c2 cells treated with H<sub>2</sub>O<sub>2</sub> (500 μM) for 0, 3, 6, 12, or 24 hours. Necrosis, PI+; Apoptosis, Annexin V+/PI-; Data are the mean ± SD (n = 4). \*p < 0.05, \*\*p < 0.01 versus control group. (C) FGF-2 significantly increased cell viability in H<sub>2</sub>O<sub>2</sub> (500 μM)-treated H9c2 cardiomyocytes. Cell viability was analyzed with a CCK8 assay. Data are the mean ± SD (n = 5). \*p < 0.05, \*\*p < 0.01 versus the Control group; #p < 0.05 versus the Model group. (D) Necrotic cells were analyzed using a PI assay in H9c2 cells exposed to FGF-2 and 500 μM H<sub>2</sub>O<sub>2</sub> for 24 hours. Data are the mean ± SD (n = 3). \*p < 0.05, \*\*p < 0.01 versus the Control group; #p < 0.05 versus the Model group. (E) Wortmannin, a PI3K inhibitor, attenuated the protective effect of FGF-2. Cell viability was measured by a CCK8 assay. Data are the mean ± SD (n = 5). \*p < 0.05, \*\*p < 0.01 versus Control group; #p < 0.05 versus the H<sub>2</sub>O<sub>2</sub> group. (F) Necrotic cells were analyzed using a PI assay in H9c2 cells exposed to FGF-2 and 500 μM H<sub>2</sub>O<sub>2</sub> with or without Wortmannin for 24 hours. Data are the mean ± SD (n = 3). \*p < 0.05, \*\*p < 0.01 versus the control group; #p < 0.05 versus the H<sub>2</sub>O<sub>2</sub> group.

after exposure to H<sub>2</sub>O<sub>2</sub> (Fig. 2A). First, H9c2 cells were treated with 500 μM H<sub>2</sub>O<sub>2</sub> for different times, and the phosphorylation of FoxO3a and Akt was determined by Western blotting. As shown in Fig. 2, H<sub>2</sub>O<sub>2</sub> decreased the phosphorylation of Akt and FoxO3a in a time-dependent manner in H9c2 cells. A significant decrease in the levels of phosphorylated FoxO3a and



**Fig. 2.** FGF-2 increased the phosphorylation of Akt and FoxO3a via the PI3K/AKT/FoxO3a pathway. Phosphorylation of Akt and FoxO3a was analyzed by Western blotting. The relative levels of p-FoxO3a versus total FoxO3a and p-Akt versus total Akt in each sample were determined with the Quantity One tool. (A) Effects of  $\text{H}_2\text{O}_2$  on the phosphorylation of Akt and FoxO3a. The data are shown as the mean  $\pm$  SD (n = 3). \*p < 0.05, \*\*p < 0.01 versus the control group. (B) The effect of FGF-2 on FoxO3a and Akt phosphorylation. Data are the mean  $\pm$  SD (n = 3). \*p < 0.05, \*\*p < 0.01 versus the control group; #p < 0.05 versus the Model group. (C) Effects of Wortmannin on the phosphorylation of Akt and FoxO3a induced by FGF-2 in H9c2 cardiomyocytes. H9c2 cardiac cells were treated with Wortmannin (1  $\mu\text{M}$ ) for 60 min prior to stimulation with FGF-2, followed by 24 hours of culture with  $\text{H}_2\text{O}_2$ . Data are the mean  $\pm$  SD (n = 3). \*p < 0.05, \*\*p < 0.01 versus the control group; #p < 0.05, ##p < 0.01 versus the  $\text{H}_2\text{O}_2$  group.

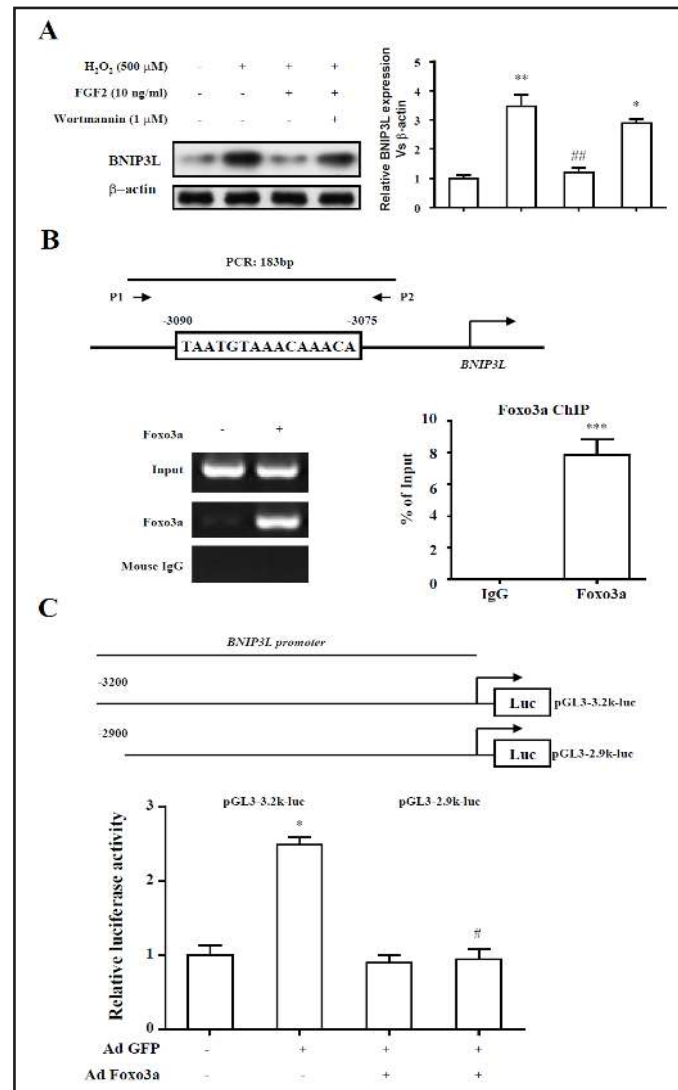
Akt was observed after 24 hours of  $\text{H}_2\text{O}_2$  treatment. To further determine the protective effects of FGF-2 against  $\text{H}_2\text{O}_2$ -induced necrosis, the levels of phosphorylated Akt and FoxO3a were measured by Western blot analysis. In Fig. 2B, the expression of phosphorylated Akt and FoxO3a was clearly reduced in the  $\text{H}_2\text{O}_2$ -treated group. Treatment with FGF-2 clearly



**Fig. 3.** FGF-2 attenuated FoxO3a translocation during H<sub>2</sub>O<sub>2</sub>-induced necrosis in H9c2 cells. The nuclear and cytosolic FoxO3a protein was analyzed by immunoblotting. The relative levels of nuclear FoxO3a versus the nuclear marker histone 3 and of cytosolic FoxO3a versus the cytosolic marker β-actin in each sample were determined using the Quantity One tool. (A) H<sub>2</sub>O<sub>2</sub> promoted FoxO3a translocation from the cytoplasm to the nucleus. The data are shown as the mean ± SD (n = 3). \*p < 0.05, \*\*p < 0.01 versus the control group. (B) The effects of FGF-2 on FoxO3a nuclear translocation. Data are the mean ± SD (n = 3). \*p < 0.05, \*\*p < 0.01 versus the Control group; #p < 0.05 versus the Model group. (C) Wortmannin inhibited the effects of FGF-2 on FoxO3a nuclear translocation. Data are the mean ± SD (n = 3). \*p < 0.05, \*\*p < 0.01 versus the control group; #p < 0.05, ##p < 0.01 vs the H<sub>2</sub>O<sub>2</sub> group.

increased the phosphorylated FoxO3a and Akt levels in H9c2 cells. Furthermore, FGF-2 treatment alone also significantly increased phosphorylated Akt and FoxO3a levels. The total Akt (t-Akt) and FoxO3a (t-FoxO3a) levels were unchanged among the four groups (Fig. 2B). To confirm the role of the PI3K/Akt/FoxO3a pathway in the protective effects of FGF-2, H9c2 cells were pretreated with the PI3K inhibitor Wortmannin before treatment with FGF-2 (Fig. 2C). Wortmannin inhibited the phosphorylation of Akt and FoxO3a compared to the FGF-2 group. These results suggested that the protective effects of FGF-2 were mediated by the PI3K/Akt/FoxO3a signaling pathway.

**Fig. 4.** FoxO3a promoted BNIP3L gene expression by directly targeting the promoter region of the BNIP3L. (A) The mRNA and protein expression of BNIP3L were analyzed using Western Blotting and realtime PCR in H9c2 cells exposed to FGF-2 and 500  $\mu$ M  $H_2O_2$  with or without Wortmannin for 24 hours. Data are means  $\pm$  SD (n = 3). \*p < 0.05, \*\*p < 0.01 versus the Control group; #p < 0.05 versus the  $H_2O_2$  group. (B) Cells transfected with or without BNIP3L were prepared for the ChIP assay. Data are the mean  $\pm$  SD (n = 3). \*\*\*p < 0.001 versus the IgG group; Schematic diagrams of the BNIP3L gene indicate the predicted binding sites. H9c2 cells were transiently co-transfected with empty or BNIP3L overexpression vectors and with luciferase-reporter constructs containing ~3.2 and ~2.9 kb of the BNIP3L promoter with or without the predicted binding site. Luciferase activity was compared to the control. The data are presented as the mean  $\pm$  SD. \*p < 0.05, versus the control group.



*FoxO3a translocation is involved in the cardioprotection against H<sub>2</sub>O<sub>2</sub>-induced necrosis in H9C2 cells by FGF-2*

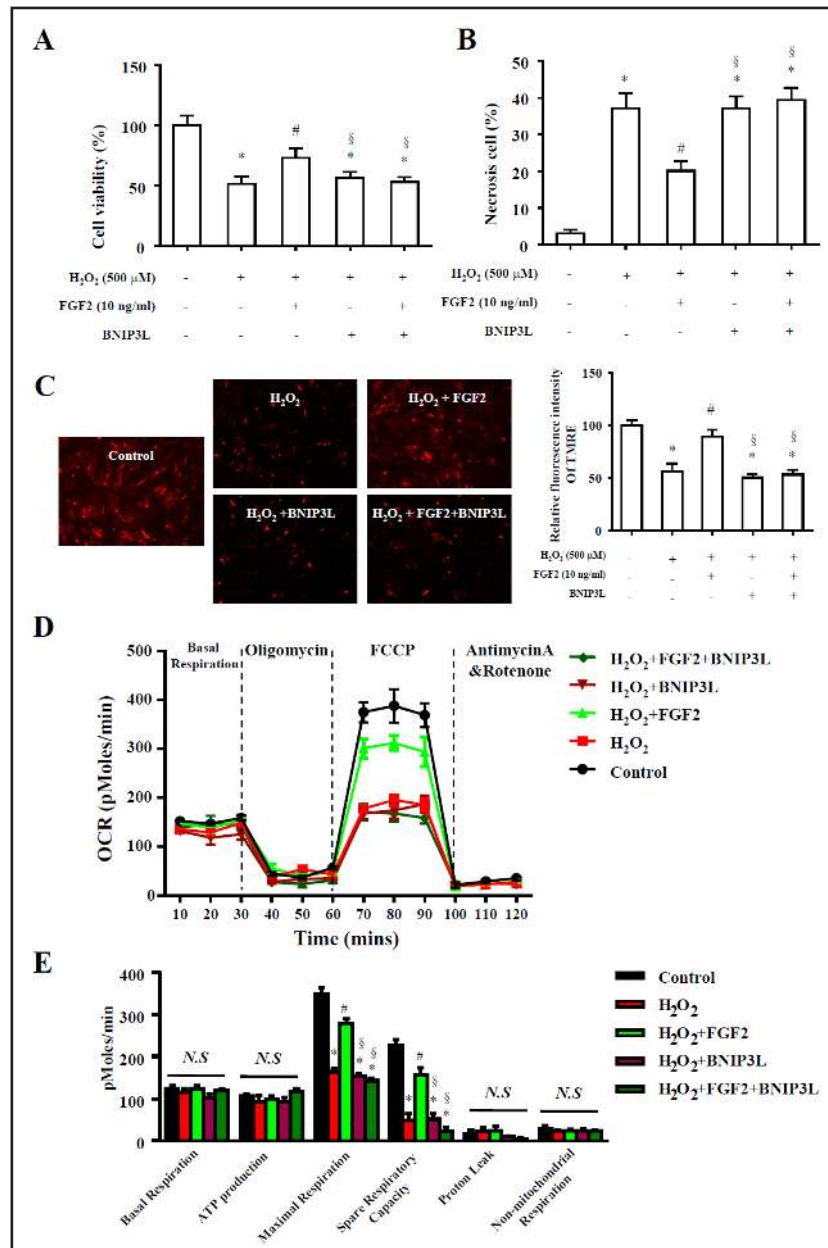
Because dephosphorylation of FoxO3a can promote Foxo3a translocation into the nucleus, we studied whether  $H_2O_2$  would promote FoxO3a translocation (Fig. 3A). We measured FoxO3a protein expression separately in the nucleus and cytoplasm using Western blot analysis. We found that FoxO3a was significantly increased in the nucleus, and the cytoplasmic level was decreased. Because FGF-2 treatment rescued the expression of p-Akt and p-FoxO3a, we further investigated whether FGF-2 was involved in the regulation of FoxO3a translocation from the cytoplasm to the nucleus after treatment with  $H_2O_2$ . We found that  $H_2O_2$  enhanced the nuclear localization of FoxO3a, whereas FGF-2 blocked the effects of  $H_2O_2$  (Fig. 3B). Furthermore, wortmannin upregulated FoxO3a in the nucleus and down-regulated FoxO3a in the cytoplasm (Fig. 3C). All of these results suggested that the protective effects of FGF-2 were mediated by the PI3K/Akt/FoxO3a signaling pathway.

*FoxO3a promoted BNIP3L gene expression by directly targeting the promoter region of BNIP3L*

As a transcription factor, FoxO3a may transcriptionally regulate some necrosis-related gene expression. To determine the possible targets of FoxO3a, we analyzed the binding sites of Foxo3a and the potential promoter region. We found a conserved consensus sequence



**Fig. 5.** Overexpression of BNIP3L in H9c2 cardiomyoblast cells abolished the cardioprotection of FGF-2 against  $H_2O_2$ -induced necrosis and mitochondrial dysfunction. (A) Cell viability was analyzed with a CCK8 assay in H9c2 cells treated with  $H_2O_2$  (500  $\mu$ M) and FGF-2 (10  $\mu$ g/ml) with or without BNIP3L overexpression. Data are the mean  $\pm$  SD (n = 3). \*p < 0.05 versus the Control group; #p < 0.05 versus the  $H_2O_2$  group; §p < 0.05 versus the  $H_2O_2$  + FGF-2 group; (B) Necrotic cells were analyzed using a PI assay in H9c2 cells exposed to FGF-2 and 500  $\mu$ M  $H_2O_2$  for 24 hours, with or without BNIP3L overexpression. Data are the mean  $\pm$  SD (n = 3). \*p < 0.05 versus the control group; #p < 0.05 versus the  $H_2O_2$  group; §p < 0.05 versus the  $H_2O_2$  + FGF-2 group; (C) Epifluorescence microscopy of H9c2 cells was assessed for mitochondrial  $\Delta\Psi_m$  indicated by TMRM (see the Methods section for



details). The intensity of fluorescence was analyzed with Image pro plus software. Data are the mean  $\pm$  SD (n = 6). \*p < 0.05 versus the control group; #p < 0.05 versus the  $H_2O_2$  group; §p < 0.05 versus the  $H_2O_2$  + FGF-2 group; (D) Mitochondrial respiration measurements of OCR were performed with a Seahorse metabolic analyzer. Oligomycin (1  $\mu$ M), FCCP (1  $\mu$ M), and rotenone (1  $\mu$ M) combined with antimycin (1  $\mu$ M) were added sequentially to H9c2 cells treated with  $H_2O_2$  (500  $\mu$ M) as well as FGF-2 with or without BNIP3L overexpression as indicated. (E) Mitochondrial function parameters (basal respiration, ATP production, maximal respiration, spare respiratory capacity, proton leak and non-mitochondrial respiration) are shown in the bar chart in Fig. 5. Data are the mean  $\pm$  SD (n = 6). \*p < 0.05 versus the control group; #p < 0.05 versus the  $H_2O_2$  group; §p < 0.05 versus the  $H_2O_2$  + FGF-2 group.

for FoxO3a binding ~3.0 – 3.1 kb upstream of the BNIP3L gene promoter (Fig. 4B). We first assessed whether BNIP3L mRNA and protein expression levels were altered after  $H_2O_2$  and FGF-2 treatment. Interestingly, the expression of BNIP3L was consistent with FoxO3a expression (Fig. 4A). This result suggested that BNIP3L may be a response gene of FoxO3a.

BNIP3L, as a potent death factor; it causes programmed necrosis by localizing to the mitochondria and endoplasmic reticulum, where it increases calcium stores and therefore the mitochondrial delivery of calcium that causes pore transition. We hypothesized that FoxO3a promoted BNIP3L gene expression through a direct interaction with the ~3.0 – 3.1 kb upstream of the BNIP3L gene promoter. A chromatin immunoprecipitation assay (ChIP) was performed. As illustrated in Fig. 4B, we found specific binding of FoxO3a to the ~3.0 – 3.1 kb region of the BNIP3L promoter, which contained the binding sequence. These data indicate that FoxO3a interacted with the binding sequence of the BNIP3L promoter in H9c2 cells. We also used a luciferase reporter construct containing the BNIP3L promoter with the ~3.2 kb region to monitor BNIP3L transcription. Overexpression of FoxO3a caused statistically significant increase in the BNIP3L promoter activity compared to the empty vector control. In contrast, overexpression of BNIP3L had no statistically significant effect on the pGL3–2.9k-luc construct, which did not contain the binding sequence (Fig. 4C). Taken together, FoxO3a promoted BNIP3L gene expression by directly targeting the promoter region of BNIP3L.

*Overexpression of BNIP3L in H9C2 cardiomyoblast cells abolished the cardioprotection of FGF-2 against H<sub>2</sub>O<sub>2</sub>-induced necrosis and mitochondrial dysfunction*

To investigate whether BNIP3L was involved in the cardioprotection of FGF-2 against H<sub>2</sub>O<sub>2</sub>-induced necrosis, BNIP3L was overexpressed before H<sub>2</sub>O<sub>2</sub> and FGF-2 treatment. We found that overexpression of BNIP3L completely abolished the protective effects of FGF-2 against H<sub>2</sub>O<sub>2</sub>-induced necrotic cell death of H9c2 cells (Fig. 5A and 5B). Based on the extensive mitochondrial and cell injuries induced by H<sub>2</sub>O<sub>2</sub> in H9c2 cells, we tested the loss of mitochondrial  $\Delta\Psi_m$  with a TMRE indicator in H<sub>2</sub>O<sub>2</sub>-treated H9c2 cells (Fig. 5C). Treatment with FGF-2 increased the TMRE  $\Delta\Psi_m$ , and this increase was completely abolished by BNIP3L overexpression (Fig. 5C). Because the transfer of electrons through electron transport chain complexes on the inner mitochondrial membrane is essential for establishing the electromotive force and proton gradient for maintaining mitochondrial  $\Delta\Psi_m$  [38], whereas mitochondrial-associated Bnip3 disrupts  $\Delta\Psi_m$  and causes programmed necrosis. We predicted that the observed loss of  $\Delta\Psi_m$  in H9c2 cells treated with H<sub>2</sub>O<sub>2</sub> might be related to a disruption in respiratory chain activity. To test this possibility, we assessed mitochondrial respiration in control cells and H<sub>2</sub>O<sub>2</sub>-treated cells with or without FGF-2 treatment or BNIP3L overexpression (Fig. 5D). Compared to the control cells, the H<sub>2</sub>O<sub>2</sub>-treated cells exhibited a marked reduction in maximal respiration, which was reflected in reduced oxygen consumption (Fig. 5E). The H<sub>2</sub>O<sub>2</sub>-treated cells had a significant loss of spare respiratory capacity, which indicated severely impaired mitochondrial respiration (Fig. 5E). As expected, treatment with FGF-2 evidently preserved the MMR and RRC, which indicated preserved mitochondrial respiration (Fig. 5D). Furthermore, overexpression of BNIP3L totally abolished the protective effect of FGF-2 on maximal respiration and spare respiratory capacity (Fig. 5E). These data indicate that the protective effects of FGF-2 are mediated by down-regulation of BNIP3L, which is the downstream gene of PI3K/AKT/FoxO3a.

## Discussion

Cardiomyocyte death is an important reason underlying cardiac syndromes, such as HF, MI and stroke. Necrosis is one of the main forms of cell death, and its role has been studied in heart diseases. Guerra et al. [9] first reported that the level of necrosis was greater than apoptosis in HF patients. Recent studies have demonstrated that necrosis can slow down and reduce cell injury in models of cardiac infarction [39]. These studies showed that necrosis has an important role in the pathological process in cardiac diseases. H<sub>2</sub>O<sub>2</sub> is a representative ROS produced during the redox process and plays a key role in intracellular signaling under pathophysiological conditions [18–22]. Given that oxidative stress is a crucial initiator of cardiomyocyte necrosis, blocking the necrosis process induced by oxidative stress may help to slow down or even prevent the onset and progression of cardiovascular disease. We therefore studied the underlying signaling pathways and molecules that mediate the effect

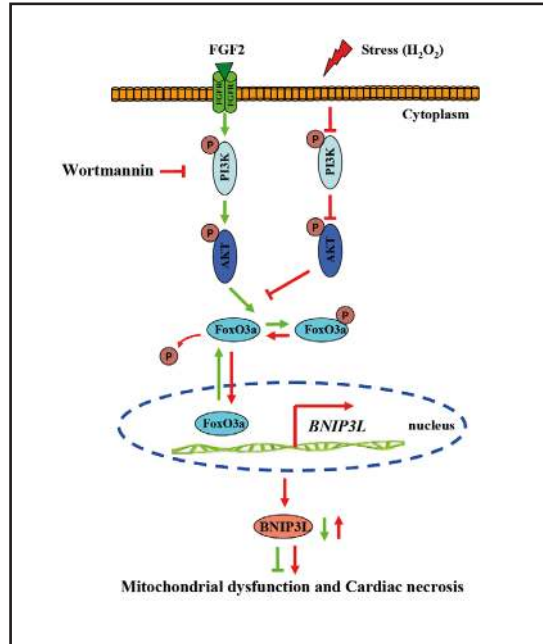
of FGF-2 to protect H9c2 cardiomyocytes against  $H_2O_2$ -induced necrosis.

The molecular mechanisms that underlie the FGF-2 rescue of cells from oxidative stress damage in cardiovascular disease remain unclear. Although several paradigms, including activation of PKC and ERK [29], inhibition of p38, inhibition of ER stress, decreased apoptosis, necrosis and autophagy, and altered gene expression [25-27] have been proposed as putative underlying mechanisms, to date none there has not been a unifying explanation to account for the cellular defects. Furthermore, additional pathways may also play a role in the mediation of the protective effects of FGF-2. In this study, we provided new, compelling evidence that  $H_2O_2$  provokes necrotic death and mitochondrial perturbation through a mechanism that involves the PI3K/AKT/FoxO3a signaling pathway. Furthermore, we revealed that BNIP3L, as a directly downstream mediator of the PI3K/AKT/FoxO3a signaling pathway, links mitochondrial respiratory defects and necrotic cell death to the oxidative stress damage.

The forkhead transcription factor Foxo3a participates in regulating diverse cellular functions, such as cell cycle arrest, oxidative scavenging, differentiation, metabolism, proliferation, hypertrophy, apoptosis and survival. However, it is currently unknown whether Foxo3a can also induce necrosis. The FoxO3a transcription factor is an important downstream target of the PI3K/Akt pathway. Activated Akt phosphorylates FoxO3a and leads to cytoplasmic localization of FoxO3a as well as inhibition of its transcriptional activity. Inhibition of the PI3K/Akt pathway increases the nuclear translocation of FoxO3a and promotes neuronal cell death.

BNIP3L, also known as NIX, is a member of the so-called BH3-only subfamily of Bcl-2 family proteins [14, 40]. The functions of Bnip3L are complicated and include cell death and mitophagy. The cell death mediated by Bnip3L with transmembrane domains that contribute more than the BH3 domain was implicated in heart disease. It was reported that recombinant NIX protein induced a loss of mitochondrial membrane potential ( $\Delta\Psi_m$ ) and cytochrome c was released from isolated mitochondria *in vitro* [41]. The molecular mechanism through which NIX induced cell death is not well understood. Collectively, there is not a single mechanism through which NIX induced cell death. Instead, there appears to be several mechanisms, and the mechanism likely depends on the cell type and experimental conditions. One model, which fits most data is that upon activation BNIP3L inserts into the mitochondrial outer membrane, and causes opening of the MPTP, loss of  $\Delta\Psi_m$ , generation of reactive oxygen species, and necrosis [42-45]. In this study, we focused on the functional significance of the PI3K/AKT/FoxO3a signaling pathway, which was down-regulated in  $H_2O_2$ -induced cardiomyocyte necrosis. The present study showed for the first time that BNIP3L is a prerequisite for  $H_2O_2$  to initiate necrosis. We demonstrated that BNIP3L acted as the downstream target of FoxO3a. BNIP3L overexpression can totally block the cardioprotection of FGF-2 against  $H_2O_2$ -induced H9c2 cell necrosis by suppressing mitochondrial function. Our results provide a new clue for the understanding of PI3K/AKT/FoxO3a-controlled cellular events and suggest that BNIP3L could be a potential therapeutic target for cardiovascular disease.

**Fig. 6.** Schematic model showing FGF-2 inhibition of  $H_2O_2$ -induced necrosis in H9c2 cells via PI3K/Akt/FoxO3a signaling, which was dependent on down-regulation of the expression of BNIP3L.



In summary, the present study showed that FGF-2 was able to inhibit necrosis in cellular models of necrosis. Our results further revealed that the PI3K/AKT/Foxo3a signaling pathway was able to regulate necrosis by transcriptionally targeting BNIP3L. The modulation of BNIP3L levels may provide an intriguing approach for preventing myocardial necrosis. Accordingly, it can be speculated that the identification of BNIP3L as a regulator of necrosis may fill in the gap between the unknown aspects of cell biology and the pathogenesis of diseases.

## Acknowledgments

This work was supported by the Innovation Program of the Shanghai Municipal Education Commission (12YZ040) and the Wu Jieping Medical Foundation (grant No. 320.6750.14322).

## Disclosure Statement

All authors declare that they have no conflicts of interest.

## References

- 1 Reed GW, Rossi JE, Cannon CP: Acute myocardial infarction. *Lancet* DOI:10.1016/S0140-6736(16)30677-8.
- 2 Hausenloy DJ, Yellon DM: Ischaemic conditioning and reperfusion injury. *Nat Rev Cardiol* 2016;13:193-209.
- 3 Bulluck H, Yellon DM, Hausenloy DJ: Reducing myocardial infarct size: challenges and future opportunities. *Heart* 2016;102:341-348.
- 4 Hu J, Lu Y: Statin and myocardial ischemia-reperfusion injury. *Int J Cardiol* 2016;222:988.
- 5 Wang X, Cao Y, Shen M, Wang B, Zhang W, Liu Y, He X, Wang L, Xia Y, Ding M, Xu X, Ren J: DIDS reduces ischemia/reperfusion-induced myocardial injury in rats. *Cell Physiol Biochem* 2015;35:676-688.
- 6 Li W, Yan S, Zhao J, Ding X, Zhang S, Wang D, Liu L, Peng W, Li H, Wang D, Liu Z, Li Y: Metoprolol Inhibits Cardiac Apoptosis and Fibrosis in a Canine Model of Chronic Obstructive Sleep Apnea. *Cell Physiol Biochem* 2015;36:1131-1141.
- 7 Rajtik T, Carnicka S, Szobi A, Giricz Z, J OU, Hassova V, Svec P, Ferdinandy P, Ravingerova T, Adameova A: Data on necrotic and apoptotic cell death in acute myocardial ischemia/reperfusion injury: the effects of CaMKII and angiotensin AT1 receptor inhibition. *Data Brief* 2016;7:730-734.
- 8 Wang A, Zhang H, Liang Z, Xu K, Qiu W, Tian Y, Guo H, Jia J, Xing E, Chen R, Xiang Z, Liu J: U0126 attenuates ischemia/reperfusion-induced apoptosis and autophagy in myocardium through MEK/ERK/EGR-1 pathway. *Eur J Pharmacol* 2016;788:280-285.
- 9 Guerra S, Leri A, Wang X, Finato N, Di Loreto C, Beltrami CA, Kajstura J, Anversa P: Myocyte death in the failing human heart is gender dependent. *Circ Res* 1999;85:856-866.
- 10 Moe GW, Marin-Garcia J: Role of cell death in the progression of heart failure. *Heart Fail Rev* 2016;21:157-167.
- 11 Du W, Pan Z, Chen X, Wang L, Zhang Y, Li S, Liang H, Xu C, Zhang Y, Wu Y, Shan H, Lu Y: By targeting Stat3 microRNA-17-5p promotes cardiomyocyte apoptosis in response to ischemia followed by reperfusion. *Cell Physiol Biochem* 2014;34:955-965.
- 12 Wang K, Liu F, Liu CY, An T, Zhang J, Zhou LY, Wang M, Dong YH, Li N, Gao JN, Zhao YF, Li PF: The long noncoding RNA NRF regulates programmed necrosis and myocardial injury during ischemia and reperfusion by targeting miR-873. *Cell Death Differ* 2016;23:1394-1405.
- 13 McCully JD, Wakiyama H, Hsieh YJ, Jones M, Levitsky S: Differential contribution of necrosis and apoptosis in myocardial ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol* 2004;286:H1923-1935.
- 14 Whelan RS, Kaplinskiy V, Kitsis RN: Cell death in the pathogenesis of heart disease: mechanisms and significance. *Annu Rev Physiol* 2010;72:19-44.

- 15 Wang K, Long B, Li N, Li L, Liu CY, Dong YH, Gao JN, Zhou LY, Wang CQ, Li PF: MicroRNA-2861 regulates programmed necrosis in cardiomyocyte by impairing adenine nucleotide translocase 1 expression. *Free Radic Biol Med* 2016;91:58-67.
- 16 Wang X, Zhang Y, Lu P, Zhang H, Li Y, Dong H, Zhang Z: PEDF attenuates hypoxia-induced apoptosis and necrosis in H9c2 cells by inhibiting p53 mitochondrial translocation via PEDF-R. *Biochem Biophys Res Commun* 2015;465:394-401.
- 17 Wang JX, Zhang XJ, Li Q, Wang K, Wang Y, Jiao JQ, Feng C, Teng S, Zhou LY, Gong Y, Zhou ZX, Liu J, Wang JL, Li PF: MicroRNA-103/107 Regulate Programmed Necrosis and Myocardial Ischemia/Reperfusion Injury Through Targeting FADD. *Circ Res* 2015;117:352-363.
- 18 Liu MH, Zhang Y, He J, Tan TP, Wu SJ, Guo DM, He H, Peng J, Tang ZH, Jiang ZS: Hydrogen sulfide protects H9c2 cardiac cells against doxorubicin-induced cytotoxicity through the PI3K/Akt/FoxO3a pathway. *Int J Mol Med* 2016;37:1661-1668.
- 19 Tavares AM, da Rosa Araujo AS, Llesuy S, Khaper N, Rohde LE, Clausell N, Bello-Klein A: Early loss of cardiac function in acute myocardial infarction is associated with redox imbalance. *Exp Clin Cardiol* 2012;17:263-267.
- 20 Gu Y, Liang Z, Wang H, Jin J, Zhang S, Xue S, Chen J, He H, Duan K, Wang J, Chang X, Qiu C: Tanshinone IIA protects H9c2 cells from oxidative stress-induced cell death via microRNA-133 upregulation and Akt activation. *Exp Ther Med* 2016;12:1147-1152.
- 21 Purushothaman S, Nair RR: Mitoprotective antioxidant EUK-134 stimulates fatty acid oxidation and prevents hypertrophy in H9C2 cells. *Mol Cell Biochem* 2016;420:185-194.
- 22 Theccanat T, Philip JL, Razzaque AM, Ludmer N, Li J, Xu X, Akhter SA: Regulation of cellular oxidative stress and apoptosis by G protein-coupled receptor kinase-2; The role of NADPH oxidase 4. *Cell Signal* 2016;28:190-203.
- 23 Wang K, Liu F, Zhou LY, Ding SL, Long B, Liu CY, Sun T, Fan YY, Sun L, Li PF: miR-874 regulates myocardial necrosis by targeting caspase-8. *Cell Death Dis* 2013;4:e709.
- 24 Golbidi S, Botta A, Gottfred S, Nusrat A, Laher I, Ghosh S: Glutathione administration reduces mitochondrial damage and shifts cell death from necrosis to apoptosis in ageing diabetic mice hearts during exercise. *Br J Pharmacol* 2014;171:5345-5360.
- 25 Powers CJ, McLeskey SW, Wellstein A: Fibroblast growth factors, their receptors and signaling. *Endocr Relat Cancer* 2000;7:165-197.
- 26 Jiang ZS, Padua RR, Ju H, Doble BW, Jin Y, Hao J, Cattini PA, Dixon IM, Kardami E: Acute protection of ischemic heart by FGF-2: involvement of FGF-2 receptors and protein kinase C. *Am J Physiol Heart Circ Physiol* 2002;282:H1071-1080.
- 27 Liao S, Porter D, Scott A, Newman G, Doetschman T, Schultz Jel J: The cardioprotective effect of the low molecular weight isoform of fibroblast growth factor-2: the role of JNK signaling. *J Mol Cell Cardiol* 2007;42:106-120.
- 28 Sedding DG: FoxO transcription factors in oxidative stress response and ageing--a new fork on the way to longevity? *Biol Chem* 2008;389:279-283.
- 29 Wu ZJ, Yu J, Fang QJ, Wang RX, Lian JB, He RL, Jiao HX, Lin MJ: Sodium Ferulate Prevents Daunorubicin--Induced Apoptosis in H9c2 Cells via Inhibition of the ERKs Pathway. *Cell Physiol Biochem* 2015;36:2121-2136.
- 30 Liu MH, Li GH, Peng LJ, Qu SL, Zhang Y, Peng J, Luo XY, Hu HJ, Ren Z, Liu Y, Tang H, Liu LS, Tang ZH, Jiang ZS: PI3K/Akt/FoxO3a signaling mediates cardioprotection of FGF-2 against hydrogen peroxide-induced apoptosis in H9c2 cells. *Mol Cell Biochem* 2016;414:57-66.
- 31 Zeng B, Li Y, Niu B, Wang X, Cheng Y, Zhou Z, You T, Liu Y, Wang H, Xu J: Involvement of PI3K/Akt/FoxO3a and PKA/CREB Signaling Pathways in the Protective Effect of Fluoxetine Against Corticosterone-Induced Cytotoxicity in PC12 Cells. *J Mol Neurosci* 2016;59:567-578.
- 32 Zhang Z, Zhang T, Zhou Y, Wei X, Zhu J, Zhang J, Wang C: Activated phosphatidylinositol 3-kinase/Akt inhibits the transition of endothelial progenitor cells to mesenchymal cells by regulating the forkhead box subgroup O-3a signaling. *Cell Physiol Biochem* 2015;35:1643-1653.
- 33 Jin CW, Wang H, Chen YQ, Tang MX, Fan GQ, Wang ZH, Li L, Zhang Y, Zhang W, Zhong M: Gas6 delays senescence in vascular smooth muscle cells through the PI3K/ Akt/FoxO signaling pathway. *Cell Physiol Biochem* 2015;35:1151-1166.

Chen et al.: FGF2 is Involved in the Hydrogen Peroxide-Induced Necrosis and Mitochondrial Dysfunction in H9c2 Cells

- 34 Zhang MQ, Zheng YL, Chen H, Tu JF, Shen Y, Guo JP, Yang XH, Yuan SR, Chen LZ, Chai JJ, Lu JH, Zhai CL: Sodium tanshinone IIA sulfonate protects rat myocardium against ischemia-reperfusion injury via activation of PI3K/Akt/FOXO3A/Bim pathway. *Acta Pharmacol Sin* 2013;34:1386-1396.
- 35 Juhasz B, Thirunavukkarasu M, Pant R, Zhan L, Penumathsa SV, Secor ER, Jr, Srivastava S, Raychaudhuri U, Menon VP, Otani H, Thrall RS, Maulik N: Bromelain induces cardioprotection against ischemia-reperfusion injury through Akt/FOXO pathway in rat myocardium. *Am J Physiol Heart Circ Physiol* 2008;294:H1365-1370.
- 36 Gurevich RM, Regula KM, Kirshenbaum LA: Serpin protein CrmA suppresses hypoxia-mediated apoptosis of ventricular myocytes. *Circulation* 2001;103:1984-1991.
- 37 Roy Chowdhury SK, Smith DR, Saleh A, Schapansky J, Marquez A, Gomes S, Akude E, Morrow D, Calcutt NA, Fernyhough P: Impaired adenosine monophosphate-activated protein kinase signalling in dorsal root ganglia neurons is linked to mitochondrial dysfunction and peripheral neuropathy in diabetes. *Brain* 2012;135:1751-1766.
- 38 Zhang P, Lu Y, Yu D, Zhang D, Hu W: TRAP1 Provides Protection Against Myocardial Ischemia-Reperfusion Injury by Ameliorating Mitochondrial Dysfunction. *Cell Physiol Biochem* 2015;36:2072-2082.
- 39 Smith CC, Davidson SM, Lim SY, Simpkin JC, Hothersall JS, Yellon DM: Necrostatin: a potentially novel cardioprotective agent? *Cardiovasc Drugs Ther* 2007;21:227-233.
- 40 Chen Y, Decker KF, Zheng D, Matkovich SJ, Jia L, Dorn GW, 2nd: A nucleus-targeted alternately spliced Nix/Bnip3L protein isoform modifies nuclear factor kappaB (NFkappaB)-mediated cardiac transcription. *J Biol Chem* 2013;288:15455-15465.
- 41 Chen Y, Lewis W, Diwan A, Cheng EH, Matkovich SJ, Dorn GW, 2nd: Dual autonomous mitochondrial cell death pathways are activated by Nix/Bnip3L and induce cardiomyopathy. *Proc Natl Acad Sci USA* 2010;107:9035-9042.
- 42 Zhang J, Ney PA: Role of BNIP3 and NIX in cell death, autophagy, and mitophagy. *Cell Death Differ* 2009;16:939-946.
- 43 Zhao M, Sun L, Yu XJ, Miao Y, Liu JJ, Wang H, Ren J, Zang WJ: Acetylcholine mediates AMPK-dependent autophagic cytoprotection in H9c2 cells during hypoxia/reoxygenation injury. *Cell Physiol Biochem* 2013;32:601-613.
- 44 Lin KH, Kuo WW, Jiang AZ, Pai P, Lin JY, Chen WK, Day CH, Shen CY, Padma VV, Huang CY: Tetramethylpyrazine Ameliorated Hypoxia-Induced Myocardial Cell Apoptosis via HIF-1alpha/JNK/p38 and IGFBP3/BNIP3 Inhibition to Upregulate PI3K/Akt Survival Signaling. *Cell Physiol Biochem* 2015;36:334-344.
- 45 Hsieh DJ, Kuo WW, Lai YP, Shibu MA, Shen CY, Pai P, Yeh YL, Lin JY, Viswanadha VP, Huang CY: 17beta-Estradiol and/or Estrogen Receptor beta Attenuate the Autophagic and Apoptotic Effects Induced by Prolonged Hypoxia Through HIF-1alpha-Mediated BNIP3 and IGFBP-3 Signaling Blockage. *Cell Physiol Biochem* 2015;36:274-284.